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Solar Radiation Damage to Human Skin Mitochondria

by

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Thesis submitted to the Dublin Institute of Technology for the degree of

Doctor of Philosophy

Supervisors: Dr. James Murphy

Dr. James Walsh



Radiation and Environmental Science Centre, Focas Institute,

Dublin Institute of Technology

November, 2010

Declaration page

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any Institute.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Signature Conclute Date 24,03.2011

"Life did not take over the globe by combat, but by cooperation"

(Witzany, 2006).

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Abbreviations

18S	Nuclear housekeeping gene region
A375	Human malignant melanoma
A549	Human lung cancer cells
AB	Alamar Blue™
AM	Air mass
ANOVA	Analysis of variances
AP1	Protein with tumour suppressive effects
ATP	Adenosine triphosphate
BCC	Basal cell carcinomas
bp	Base pair
C2C12	Non-invasive C2C12 myoblasts
C32	Human amelanotic melanoma
cDNA	Copy DNA
CF	Nuclear single copy gene of cystic fibrosis
CO ₂	Carbon dioxide
COX-2	Cyclooxygenase-2
Cyt b	Cytochrome b mitochondrial gene
DCF	Dichlorofluorescein
DMEM	Dulbecco's modified essential medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleosides
Drp1	Dynamin-related Protein 1
ECACC	European collection of cell cultures
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FACS	Fluorescence associated cell sorter
FBS	Foetal bovine serum
	Mesoxalonitrile
FCCP	trifluoromethoxyphenylhydrazone
FGF-2	Fibroblast growth factor-2
FITC	Fluorescein-isothiocyanate
FMNH2	Flavin mononucleotide
GSH	Glutathione
GTPases	Guanosine triphosphate (GTP) hydrolase
H_2O_2	Hydrogen peroxide
НаСаТ	Normal human keratinocyte cell line

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HD	High density
Kb	Kilobases
LD	Low density
LR-PCR	Long range polymerase chain reaction
LTG	LysoTracker Green
MEME	Minimal Essential Medium eagle
Mg	Magnesium
MM	Mitochondrial mass
MMP	Mitochondrial membrane potential
MnSOD	Manganese superoxide dismutase
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
mtDNA ³⁸⁹⁵	Mitochondrial DNA deletion -3895 bp
mtDNA ⁴⁹⁷⁷	Mitochondrial DNA deletion -4977 bp
MTP	Mitochondrial transition pore
MTR	Mitotracker Red
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-
	diphenyltetrazoliumbromid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide dehydrogenase
ND1	NADH dehydrogenase 1
ND5	NADH dehydrogenase 5
nDNA	Nuclear DNA
NMSC	Non-melanoma skin cancer
NO	Nitric oxide
NOS	Nitric oxide species
OPA1	Optic Atrophy 1
OXPHOS	Oxidative phosphorylation
p53	Tumour suppressor gene
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's Disease
Pd(N)6	Random hexamer
Pi	Inorganic phosphate
PI	Propidum iodide
pol B	DNA polymerase β gene
PTP	Permeability transition pore
QPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reactions
RFU	Relative Fluorescent Units
RNA	Ribonucleic acid
ROI	Region of interest

ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Real Time PCR
SCC	Squamous cell carcinomas
SD	Standard deviation
SDS	Sodium dodecylsulphate
SSR	Simulated solar radiation
ТСА	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
TMRM	Tetramethyl Rhodamine Methyl Ester
tRNA	Transfer RNA
t-test	Student's t-test
UV	Ultraviolet
UVA	Ultraviolet-A
UVB	Ultraviolet-B
UVC	Ultraviolet-C
UVR	Ultraviolet radiation
Ψm	Deltapsi(m) membrane potential
ΨРМ	Plasma membrane potential
ΨΜΤ	Mitochondrial Membrane Potential

Abstract

The central objective of this study was to assess solar radiation-induced changes in cellular function, mitochondrial function and mitochondrial DNA to further investigate the role of these energy-dedicated and metabolically essential organelles in the response to the main environmental stressor associated with skin cancer initiation.

An *in vitro* approach was chosen employing the human malignant melanoma (A375) and human amelanotic melanoma (C32) cells and the human spontaneously immortalized keratinocytes (HaCaT). A Q-Sun Solar Simulator was used to expose cells to low-level simulated solar radiation (SSR) as it provides a mimic of solar radiation that is environmentally relevant in the UV spectrum. Cell viability, apoptosis, DNA and protein content were analysed as cellular response end-points and they have been observed to change in a cell type-specific and time- dependent manner post SSR.

Increases in mitochondrial genome number and mtDNA³⁸⁹⁵ were observed as an early response to low-dose SSR in human skin cells. The common deletion mtDNA⁴⁹⁷⁷, though detected, did not directly increase in frequency with solar radiation exposure though the mtDNA³⁸⁹⁵ deletion, previously found to be associated with solar radiation exposure, was observed to be substantially increased in a cell-type and dose-dependent manner in skin cells post SSR.

Impaired mitochondrial bioenergetics, dynamics and recycling may play a significant role in the melanoma tumour initiation and progression in humans post systematic solar radiation over-exposure. Furthermore the sensitive nature of the mitochondrial population of skin cells should not be underestimated as dynamic changes in their biology are evident even in cell populations that received low level irradiation of simulated solar radiation.

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Chapter 1 - Introduction

1.1 Solar Radiation and skin carcinogenesis

The mechanism by which the biological systems tolerate and react to environmental stress has been the subject of intense research in the biomedical sciences for many years. Solar irradiation, as one of the most important environmental stressors, has long been known to be the main player in the etiological cause of most skin cancers and accelerated skin ageing (Setlow *et al.*, 1993; de Gruijl, 1999; Moan *et al.*, 1999).

The electromagnetic radiation spectrum encompasses an extensive range of wavelengths, quantised as photons (Cotterrill, 2002). The sun emits a spectral continuum ranging from approximately 280 nm to 4000 nm and peaking at about 2 W/m² in the green region of the visible waveband around 550 nm. This spectral continuum has been subdivided, according to the Commission Internationale d'Eclairage (CIE), into three convenient photobiological wavebands which are UVC, 100 – 280 nm, UVB, 280- 315 nm and UVA, 315 – 400 nm (Akbar-Khanzadeh and Jahangir-Blourchian, 2005). A standard solar spectrum is not available as environmental factors such as season, latitude, time of the day, atmospheric content and cloud cover exert direct influence on the attenuation of the extraterrestrial solar spectrum.

Shorter solar wavelengths possess greater energy and have a higher biological toxicity which has been demonstrated both epidemiologically and clinically (Reme et al., 1996; de Gruijl, 1997; Matsumura and Ananthaswamy, 2004; Glanz and Mayer, 2005). As

almost all of the extraterrestrial solar UVC is absorbed in the atmosphere, the most photo-biologically active solar waveband is the UVB which comprises 5-10% of the total terrestrial solar UVR (Monroy et al., 1998; Diffey, 2000). UVB radiation induces sunburn and skin pigmentation and cell damage with greater efficiency than UVA, although biological effects of UVA such as skin tanning or 'melanogenesis' and photoageing should not be underestimated (Sabburg et al., 2001; Moyal, 2004; Elsner et al., 2007; Fleming, 2008). It has been reported that UVA penetrates the skin more deeply than UVB in the dermis and can cause greater vascular insult, while UVB is almost completely absorbed in the epidermis (Grant et al., 2003; Reichrath, 2006).

The action spectrum defines the relative efficacy of shorter wavelength electromagnetic radiation in eliciting a pre-defined and tissue-specific biological response (Parrish et al., 1982; Scourfield and Bodeker, 2000; De Fabo, 2006). The standard and most commonly referenced action spectrum is the human erythemal action spectrum (Parisi and Wong, 1997). The erythemal effectiveness of monochromatic UVB varies from wavelength to wavelength (Gasparro, 2000). Another referenced action spectrum is known as the Minimum Erythemal Dose (MED) which can be described as the radiant exposure of UVR which produces a noticeable reddening of skin within well-defined borders, 24 hours after irradiation (Moehrle et al., 2003). Values of 200 – 300 J/m² effective correspond to 1 MED for white skin (World Health Organization, 1995).

Skin cancer is the most common form of malignancy in the Caucasian population (Diepgen and Mahler, 2002) and it is well established that its initiation is associated with Ultra Violet Radiation (UVR) over-exposure (Soehnge *et al.*, 1997). Solar UVR is implicated in the induction of both malignant melanomas and non-melanoma skin

cancer (Albino *et al.*, 2004; De Vriesmand Coebergh, 2004). Solar UV radiation (UVR) is also known to induce cellular and genomic toxicity, reactive oxygen species (ROS) formation, DNA damage, sunburnt cells and higher proteolitic rates (Danno and Horio, 1987; Murphy *et al.*, 2001; Trautinger, 2001; Chouinard *et al.*, 2002; Agar *et al.*, 2004; Karakoula *et al.*, 2005; Kowalczu *et al.*, 2006; Yang *et al.*, 2006). If the cellular repair and antioxidant responses to UVR are overwhelmed, cellular photodamage can occur and may initiate photocarcinogenesis (Shindo *et al.*, 1994; Nishiyama *et al.*, 2001; Sander *et al.*, 2004).

One of the most serious effects of UV is DNA mutagenesis (Smith *et al.*, 1995; Kasai, 1997; Kielbassa *et al.*, 1997). UVR absorption by DNA is potentially mutagenic and may lead to the formation of DNA photo-products which are associated with mutagenesis and cancer initiation (Kvam and Tyrrell, 1997). Furthermore, DNA damage can be indirectly initiated by generation of solar radiation-induced ROS (de Gruijl *et al.*, 2001). Solar radiation-induced DNA strand breaks and protein cross-links are considered direct effects whereas increased mitochondrial damage and/or dysfunction associated with augmented ROS formation, lipid and protein oxidation and energy depletion are considered to be indirect effects of UVR (Ichihashi *et al.*, 2003).

It has been demonstrated that visible light can induce cellular dysfunction and cell death both in *in vitro* and *in vivo* models (Tyrrell *et al.*, 1984; Jones *et al.*, 1987; Lai *et al.*, 1987; Setlow and Woodhead, 1994; Peak and Peak, 1995). Damaged cells are eliminated as a way of protecting the organism from the possible tumourigenic transformation risk represented by damaged cell accumulation. Additionally, cell death post solar radiation is important in forming a protective layer of dead cells, the stratum corneum, which is an

extra UVR-protective mechanism at the organism level (de Winter *et al.*, 2001; Mass *et al.*, 2003). Time-course studies revealed that the maximal accumulation of UV-induced cell death (sunburnt cells) occurs as early as 8 hours post UVR irradiation and are maximally expressed 24-48 hours post photodamage in keratinocytes (Woodcock and Magnus, 1976; Danno and Horio, 1987).

The two main types of cell death induced by solar radiation are apoptosis and necrosis. The former involves a cascade of events leading to progressive fragmentation and vacuolization of the cell whereas the latter occurs mostly as a passive process involving cell swelling and disruption of the cell membrane. In addition, while necrosis always affects groups of cells, apoptosis is mostly seen in isolated events. (Wooscock and Magnus, 1976; Godar, 1996; Hirsch *et al.*, 1997; Ono *et al.*, 2003).

UV-induced apoptosis is recognized as a protective mechanism avoiding the malignant transformation through negative selection of severely damaged cells. Solar radiationinduced apoptosis is a complex process involving multiple molecular pathways including DNA damage accumulation, mitochondrial damage and cytochrome c release as well as the activation of tumour suppressor gene p53 (Yamaizumi and Sugano, 1994; Godar and Lucas, 1995; Sun *et al.*, 1998; Goldstein *et al.*, 2000; Roos and Kaina, 2006). During apoptosis, the mitochondrial network is disintegrated and the outer mitochondrial membrane permeabilized, which results in the release of several apoptogenic proteins, including cytochrome c (Breckenridge *et al.*, 2003).

Apoptosis deregulation is often involved in neoplasic transformation. Indeed, p53 mutations, similar to the ones induced by UVR, are present in over 90% of squamous cell

carcinomas and more than 50% of basal cell carcinomas (Brash *et al.*, 1991; Rady *et al.*, 1992; Campbell *et al.*, 1993). In addition, mitochondria play a central role in initiating and controlling apoptotic pathways (Susin *et al.*, 1998; Ferri and Kroemer, 2001).

UVR may elicit cellular stress responses, interfering with cell cycle regulation, allowing extra time for cellular repair or inducing cell proliferation (McGregor *et al.*, 1991; Manke *et al.*, 2005). Additionally, tumour cells have long been reported to heavily rely on glycolysis instead of oxidative phosphorylation as the main energy production pathway (Warburg effect) (Warburg, 1956). An association between programmed cell death evasion and the glycolytic shuttle has also been hypothesised although mechanistic confirmation remains to be described (Weinhouse, 1956; Xu and Guppy, 2004; Kim and Dang, 2006).

Cellular differentiation and gene expression can also interfere with the outcome of solar radiation-induced cellular damage. For instance, post UVR irradiation, basal keratinocytes repair damaged DNA whereas differentiating keratinocytes undergo cell death, both processes strictly controlled by p53 (Campbell *et al.*, 1993; Norris *et al.*, 1997; Young *et al.*, 1998; D'Errico *et al.*, 2006). Solar radiation can induce up-regulation of several proteins involved in coping with oxidative and cell damage such as antioxidants, DNA repair proteins, heat shock proteins and proteins associated with energy production (Farrell *et al.*, 1998; Saliou *et al.*, 1999; Morris, 2002; Pisarchik *et al.*, 2004).

Organelles such as mitochondria can be targeted by UVR-induced ROS and suffer functional disruption and genotoxic effects, such as deletions and point mutations

(Berneburg *et al.*, 1997; Durham *et al.*, 2003; Eshaghian *et al.*, 2006). Additionally, depletion of mtDNA has been reported to alter gene expression profile, with cellular effects similar to that observed in photo aged skin (Schroeder *et al.*, 2008). Such a scenario can get worse due to accumulation of genetic, transcriptional and translational errors induced by solar radiation-induced oxidative stress.

Solar radiation-induced damage to mitochondria has been used as a sensitive marker for apoptosis as initiation of apoptotic cell death leads to mitochondrial permeability transition that ultimately leads to cytosolic release of cytochrome *c* (Kluck *et al.*, 1997; Gao *et al.*, 2001). Conversely, where solar radiation does not elicit apoptosis, oxidative stress elicit mitochondrial calcium influx with associated opening of the mitochondrial permeability pore (MTP) and mitochondrial membrane depolarization, resulting in mitochondrial dysfunction or up-regulation of mitochondrial proteins (Mendez and Penner, 1998; Gallo *et al.*, 1989; Gonzalez *et al.*, 2005). Cytochromes, NADH dehydrogenase, mitochondrial ATP synthase and mitochondrial ribosomal protein are amongst the mitochondrial proteins up-regulated in response to UV-irradiation (Leccia *et al.*, 2001).

1.2 Reactive Oxygen Species

ROS cause oxidative DNA damage, protein and lipid peroxidation and, above a certain threshold, stimulate apoptosis (Morliere *et al.*, 1995; Yakes and Van Houten, 1997; Sander *et al.*, 2002). Indeed, several physiological and pathological processes are associated with ROS over-production including cancer (Urbach, 1989; Berneburg *et al.*, 2000), aging (Finkel and Holbrook, 2000), neurodegenerative diseases (Simonian and Coyle, 1996), and inflammatory disorders (Brown *et al.*, 2008).

ROS are generated at low levels during normal metabolism, and have been shown to play important roles as secondary messengers in activating transcription factors and promoting cell growth and differentiation (Tatla *et al.*, 1999, Sauer *et al.*, 2001; Brown *et al.*, 2004). ROS and specifically nitric oxide (NO) are also known to interfere with cellular differentiation and to play a key role in cell division/self renewal signalling (Zuliani *et al.*, 2005; Kovacs *et al.*, 2009).

Several studies have demonstrated that irradiation of mammalian cells with visible light induced cellular damage primarily via ROS formation (Lavi *et al.*, 2003; Gonzales Maglio *et al.*, 2005; Lavi *et al.*, 2010). ROS are also generated as a result of increased metabolism of transformed cells, immune reaction against the developing tumour, melanin production and an altered antioxidant system, possibly playing an important role in tumour initiation and progression (Scharffetter-Kochanek *et al.*, 1997; Tsukahara *et al.*, 2003; Costin and Hearing, 2007).

UVR stimulates ROS synthesis which has been implicated in mutagenesis and photoageing (Adelman *et al.*, 1988; Herrling *et al.*, 2003; Sander *et al.*, 2004). Photoageing is the superposition of chronic UV-induced damage on intrinsic ageing and accounts for most age-associated changes in skin appearance. It is triggered by receptor-initiated signalling, mitochondrial damage, protein oxidation and telomere-based DNA damage response (Elder *et al.*, 1991; Fisher *et al.*, 2002; Sander *et al.*, 2002; Berneburg *et al.*, 2004; Kosmadaki and Gilchrest, 2004). Although the fundamental mechanisms are still poorly understood, a growing body of evidence points toward the involvement of

multiple pathways in the generation of aged skin (Quan *et al.*, 2002; Rittie and Fisher, 2002).

It has been suggested that cancer cells exhibit increased intrinsic ROS stress, due, in part, to oncogenic stimulation, increased metabolic activity and mitochondrial malfunction (Pearse *et al.*, 1986; Birch-Machin, 2005; DeBerardinis *et al.*, 2008). Since the mitochondrial respiratory chain is a major source of ROS generation in the cells and that the naked mtDNA molecule is in close proximity to the source of ROS, the vulnerability of the mtDNA to ROS-mediated damage appears to be a mechanism to amplify ROS stressing in cancer cells (Kang and Hamasaki, 2002; DeBerardinis *et al.*, 2008).

Additionally, mitochondrial activity is essential for UVR stimulation of ROS production (Boveris *et al.*, 1972; Shindo and Hashimoto, 1998; Gniadecki *et al.*, 2000). UVR-induced changes in intracellular redox state may also affect cellular homeostasis causing imbalance between pro and antioxidants (Dalle-Carbonare and Pathak 1992; Petersen *et al.*, 2000) which in turn, is known to be critical in regulating cell survival post UVR stress (Chung *et al.*, 2003).

Mitochondrial dysfunction and subsequent oxidative stress are suspected to contribute to many human diseases and disorders including cancer. ROS production has been associated with nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) mutagenesis (Liu *et al.*, 1996; Yakes and van Houten, 1997). ROS can damage mtDNA by inducing oxidized base formation, by increasing the proportion of both point mutation and mtDNA largescale deletions (Gadaleta *et al.*, 1992; Wei, 1992; Hayakawa *et al.*, 1992; Shigenaga *et al.*,

1994; Phillipson *et al.*, 2002). Indeed, several studies have suggested that mtDNA is more prone to ROS oxidative damage than the nDNA (Schipper *et al.*, 1998; Sawyer and van Houten, 1999).

Mitochondrial ROS are generated due to a leakage of electrons from respiratory chain activity, especially from complexes I and III (Turrens, 2003). The respiratory chain is located in the mitochondrial inner membrane, in close proximity to mtDNA molecules (Harman, 1956; Miquel et al., 1980; Shigenaga et al., 1994). Gross mitochondrial changes are seen in association with cancer (Kroemer et al., 1998) and oxidative stress caused by respiratory defects through an inhibition of complex IV (Ishii et al., 2005). Abnormal mitochondrial manganese superoxide dismutase (MnSOD) activity has been found in cancer cells (Oberley and Buettner, 1979) as well as up-regulation of tumour suppressor proteins like AP1 (Zhao et al., 2001). Furthermore, ROS such as the hydroxyl radical, superoxide anion, and singlet oxygen can be produced when visible light excites cellular photosensitizers (Scharffetter-Kochanek et al., 1997; Wondrack et al., 2004). Possible photosensitizers include flavin containing oxidases the cytochrome system, heme containing proteins and tryptophan-rich proteins (Morliere et al., 1991; Godar et al., 1993). Endogenous chromophores participate in the photoproduction of ROS which may oxidize proteins, lipids, cellular organelles and protein complexes involved in macromolecules turn-over (Hao and Fong, 1999; Wondrak et al., 2006). Recently, it has been reported that proteins from the extracellular matrix are sensitizers of photooxidative stress in human skin cells (Wondrak et al., 2003). Additionally, proteasome and lysosome damage have been reported post solar radiation, culminating in decreased degradation of oxidized proteins which can, in their turn, cause metabolic damage (Rapić-Otrin et al., 2002).

1.3 Mitochondria and cancer

Mitochondria are essential organelles that play a role in fundamental cellular process such as oxidative phosphorylation, calcium signaling and apoptosis (Pullman *et al.*, 1960; Rossi and Lehninger, 1964; Lehninger *et al.*, 1978; Adhami *et al.*, 2003). They contain a compact genome which encodes a limited number of essential mitochondrial respiratory proteins as well as the rRNAs and tRNAs necessary for intra-mitochondrial translation (Robberson *et al.*, 1972; Anderson *et al.*, 1981). In addition, mitochondria are semi-autonomous organelles that perform an essential function in the regulation of cell death, signaling and ROS generation and scavenging (Suschek *et al.*, 2001; Adhami *et al.*, 2003; Duranteau *et al.*, 1998).

Mitochondria are also essential in processing important metabolic intermediates in various pathways like carbohydrates, amino acids and fatty acids (Dubuy and Hesselbach, 1958; Klingenberg and Pfaff, 1968; Newsholme and Crabtree, 1976). These organelles owe their unique nature to their endosymbiotic origin that includes the ability to divide independently from the cell (Altmann, 1894; Dalton, 1951; Margulis, 1975; Aufderheide, 1975).

Nevertheless, mitochondrial function involves interplay between mitochondrial and nuclear genomes (Poyton and McEwen, 1996; Gleyzer *et al.*, 2005). More recently, new surprising mitochondrial characteristics have been described, as the possibility of intermitochondria exchange (Nakada *et al.*, 2001) and the existence of mitochondrial 'damage'-associated molecular patterns (DAMPs) which have been hypothesized as having an inter-cell and systemic signaling roles (Zhang *et al.*, 2010).

Mitochondrial dysfunction and DNA damage has been associated with a wide range of human disorders including mitochondrial disease and many cancer types, such as skin cancer (Rodust *et al.*, 2009) and indeed Alzheimer's disease (Pavlov *et al.*, 2010). Growing evidence suggests that cancer cells exhibit increased intrinsic mitochondrial stress, due, in part, to oncogenic stimulation, increased metabolic activity and mitochondria-nucleus signalling malfunction (DeBerardinis *et al.*, 2008; Czarnecka *et al.*, 2010). Several authors suggest that during the tumourigenic process the use of glycolytic pathway is a need of cancer cells rather than a consequence of mtDNA mutation (Xu *et al.*, 2005; Brandon *et al.*, 2006).

The cellular and mitochondrial response to UVR is understood to be cell type and tissue specific, and associated with physiological, morphological and biochemical adaptations of cellular biology (Gallo *et al.*, 1989; Ouhtit *et al.*, 2000; Wunderlich *et al.*, 2008). Mitochondria have been implicated in the carcinogenic process because of their role in apoptosis (Chomyn and Attardi, 2003) and the presence of mtDNA somatic mutations in a wide variety of human tumours (Petros *et al.*, 2005).

The respiratory dysfunction, or energetic impairment, in cancer cells has been reported by Otto Warburg (and termed the Warburg effect) who proposed that defects in energy metabolism, specifically in mitochondria, may play an important role in the initiation or progression of cancers (Warburg, 1956). Indeed, a shift in glucose metabolism from Oxidative Phosphorylation (OXPHOS) to glycolysis has been frequently observed in cancer cells and represents an important biochemical hallmark of tumours (De Bersaques, 1974; Pearse and Marks, 1978).

Due to the usual finding of reduced content of mitochondrial respiratory enzyme complexes in cancers, it has been suggested that the metastases and recurrence of cancers may be linked to the down-regulation of the mitochondrial OXPHOS system (Simonnet *et al.*, 2002). Moreover, the mitochondria-to-nucleus retrograde signaling could induce aggressive and invasive phenotypes and resistance to apoptosis (Amuthan *et al.*, 2001; Biswas *et al.*, 2005).

Petersen *et al.* (2004) found that tumours that exhibited the most pronounced "Warburg effect" had functional mitochondria with the capacity to make ATP, but the mitochondrial content was reduced in cancer cells resulting in a net decrease in oxygen consumption capacity. The p53 protein was recently shown to regulate mitochondrial respiration [Matoba *et al.*, 2006] and glucose metabolism (Kawauchi *et al.* 2008). Brain and breast tumour cells with depleted mtDNA (by treatment with ethidium bromide [rho⁰]) presented decreased tumourigenic phenotype and increased sensitivity to cytotoxic drugs indicating that mitochondria and mtDNA play a direct role in modulating aspects of the tumourigenic phenotype (Yu *et al.*, 2007; Kulawiec *et al.*, 2008).

1.4 Mitochondrial DNA alterations in human skin cancers

Human mtDNA is a 16,569 bp double-stranded circular DNA molecule, and between several hundred to several thousand copies of mtDNA are typically present in a cell. Human mtDNA encodes 13 polypeptides, which are essential constituents of respiratory enzyme complexes, and 22 transfer RNAs and two ribosomal RNAs that are required for protein synthesis in mitochondria (Anderson *et al.*, 1981).

MtDNA is both polyploid and heterogenenous in nature, and one cell may contain highly organized networks encompassing anywhere from tens to hundreds of organelles in the same cell (Bernardi, 1977). mtDNA can be distinguished from nDNA for having a higher rate of mutation, use of a divergent genetic code, transmission by maternal inheritance, the phenomenon of polyploidy and above all, specific organization and expression machinery (Giles *et al.*, 1980; Fernandez-Silva *et al.*, 2003).

MtDNA is more susceptible to oxidative damage and has a higher mutation rate compared with nDNA due to a lack of protective histone proteins, limited DNA repair activities, and a high rate of ROS generation in mitochondria (Sawyer and Van Houten, 1999). Somatic mutation and damage to mtDNA can result in impairment of the OXPHOS system and enhanced ROS production.

Mitochondria are classicially described as having little or no DNA damage repair mechanisms, based on initial studies in which mtDNA damage have been superestimated due to oxidation of mtDNA during the DNA isolation steps (Richter *et al.*, 1988; Helbock *et al.*, 1999; Santos *et al.*, 2006).

In fact, mitochondria are unable to repair some mtDNA damage such as pyrimidine dymers (LeDoux *et al.*, 1992). More recently, substantial repair of mtDNA was reported post UVC-induced lesions (Kalinowski *et al.*, 1992). It has also been suggested that mtDNA repair pathways may constitute a cellular defence mechanism against oxidative stress (Grishko *et al.*, 2005).

MtDNA repair and ROS defences have been suggested to be controlled, at least in part, by the circardian clock (Eckel-Mahan and Sassone-Corsi, 2009). Pro and anti-oxidants have been found to be produced periodically in multiple organisms (Hardeland *et al.*, 1999; Hardeland *et al.*, 2000). In addition, the expression of a protein conferring UVBresistance has recently been described to be regulated in a circadian fashion, with a peak just before the beginning of the day (Kucho *et al.*, 2005).

1.5 Mitochondrial DNA and cancer

MtDNA damage may result in defects in mitochondrial function which has long been suspected to contribute to the development and progression of cancer (Gause, 1969; Shay and Werbin, 1987; Bandy and Davison, 1990; Kalckar, 1991). Apart from causing depleted energy production and increased ROS production, mtDNA alterations can also result in inefficient or truncated mtDNA genetics expression, which can be potentially harmful to human health (Harman, 1956; Delettre *et al.*, 2000). As early as 1920, it has been hypothesised that the development of impaired cellular respiratory machinery would account for an increase in glycolytic ATP production which in turn could be a key event in the carcinogenic process (Warburg, 1956; Toyokuni *et al.*, 1995; Carew and Huang, 2002; Czarnecka *et al.*, 2006).

In recent years there has been re-emerging interest in the investigation of the mechanisms by which mitochondrial dysfunction and mtDNA mutations influence the pathophysiology and progression of cancers (Chinnery *et al.*, 2002; Petros *et al.*, 2005; Chatterjee *et al.*, 2006; King *et al.*, 2006; Hoang *et al.*, 2007). Indeed, many types of human malignancy such as colorectal, liver, breast, lung, prostate, skin and bladder cancer have been shown to harbour somatic mtDNA mutations (Hibi *et al.*, 2001; 14

Nishikawa *et al.*, 2001; Matsuyama *et al.*, 2003; Durham *et al.*, 2003; Petros *et al.*, 2005). Accumulation of mtDNA mutation is correlated with tumour aggressivity and multi-drug resistance (Lee *et al.*, 2004; Xu *et al.*, 2005) or with poor prognosis and acute symptoms and signs (Shoffner *et al.*, 1990; Harding *et al.*, 1992).

Mitochondria may be involved in a host of different aspects of tumorigenesis, including mutagenesis, maintenance of the malignant phenotype, and control of apoptosis (Cavalli and Liang, 1998; Gottlieb and Tomlinson, 2005; Zhou *et al.*, 2007). Moreover, the mitochondrial pathway of apoptosis is the major way in which vertebrate cells die during development, homeostasis and ageing and upon physiological and pathological states (Ravagnan *et al.*, 2002). Additionally, it has been demonstrated that activation of oncogenes or mutation of tumor suppressor genes, such as p53, can lead to the up regulation of glycolytic enzymes or inhibition of the biogenesis or assembly of respiratory enzyme complexes such as cytochrome *c* oxidase (Vousden and Lane, 2007; Bensaad and Vousden, 2007).

In the past decade, various types of mtDNA alterations have been identified in primary human cancers. These mtDNA alterations include point mutations, deletions, insertions, tandem duplications, and copy number change. High frequency of mtDNA somatic mutations occur in various types of cancers, and many of the mtDNA mutations are located in the D-loop region (Lee *et al.*, 2004). Large-scale deletions of mtDNA have been detected in various types of cancers: breast (Parrella *et al.*, 2001); head and neck (Dasgupta *et al.*, 2010); lung (Matsuyama *et al.*, 2003); renal cell carcinoma (Horton *et al.*, 1996); thyroid (Maximo *et al.*, 2002). Somatic mutation and mtDNA damage can result in impairment of the OXPHOS system and enhanced ROS production. This

scenario has been proposed to be involved in carcinogenesis [Chatteriee *et al.*, 2006; Fukui and Moraes, 2008; Kulawiec *et al.*, 2009].

MtDNA mutation-elicited respiratory chain deficiency could result in over-production of ROS. ROS-induced oxidative stress is involved in the expression and regulation of nuclear genes related to carcinogenesis (Sen and Packer, 1996; Allen and Tresini, 2000). Bandy and Davison (1990) hypothesized that damage to mtDNA would increase the steady-state concentration of reduced intermediates of the respiratory chain leading to the formation of free radicals from their auto-oxidation. Using hybrid technology to replace the endogenous mtDNA in tumor cells, Ishikawa *et al.* (2008) demonstrated that ROS-generating mtDNA mutation can enhance metastatic potential of tumor cells. Indeed, mtDNA damage has been found to be a good biomarker of oxidative stress in many different cell types including fibroblasts, endothelial cells, epithelial cells, retinocytes and cells of neuronal origin (Deng *et al.*, 1999; Salazar and van Houten, 1997; yakes and van Houten, 1997; Godley *et al.*, 2005; Ballinger *et al.*, 1999).

1.6 Solar radiation and mitochondrial DNA damage

Several groups have shown that deletions of mtDNA as opposed to nDNA may be useful as a biomarker of UVR exposure (Ray *et al.*, 2000; Krishnan *et al.*, 2004; Eshaghian *et al.*, 2006). Mutations are typically heteroplasmic and the proportion of mutant molecules can vary between tissues and with age (Holt *et al.*, 1989; Hirano *et al.*, 1997; Chinnery *et al.*, 2000, Wang and Boles, 2006).

Instability in the D-loop region of mtDNA, together with the decrease in mtDNA copy number, has been linked to human carcinogenesis (Chinnery *et al.*, 2000; Chatterjee *et* 16

al., 2006). Conversely, increased mtDNA content was associated with drug resistance in head and neck cancer cells (Mizumachi *et al.*, 2008). Amuthan *et al.* (2001) showed that partial depletion of mtDNA or treatment with mitochondrial specific inhibitors induced invasive phenotype in non-invasive C2C12 myoblasts and human lung cancer A549 cells. Causative mechanisms for mtDNA content variation effects upon the phenotype remain poorly understood in most disease models.

It is suggested that mtDNA point mutations can be the cause of multiple large-deletion mutant DNA and depletion of mtDNA (Mita *et al.*, 1990; Manfredi *et al.*, 1997; Maximo *et al.*, 2001). Durham *et al.* (2003) detailed investigated the entire spectrum of large-scale deletions, the incidence of common deletion and tandem duplications as well as the distribution of single base changes in the mitochondrial genome of basal cell and squamous cell carcinoma biopsies providing a detailed study of the distribution of multiple forms of mtDNA damage in non-melanoma skin cancers and in perilesional tissues.

Up to now, the most studied mutation in skin was the mtDNA⁴⁹⁷⁷, also called the 'common deletion', which has been considered to be a marker for mutations in the mitochondrial genome (Schon *et al.*, 1989; Shoffner *et al.*, 1989; Cortopassi *et al.*, 1992; Pang *et al.*, 1994; Berneburg *et al.*, 1999). mtDNA⁴⁹⁷⁷ is one of the common mtDNA mutations detected in ageing human tissues (Cortopasi *et al.*, 1992; Simonetti *et al.*, 1992; Melov *et al.*, 1995). The same mtDNA⁴⁹⁷⁷ was repeatedly described in association with skin cancer (Birch-Machin *et al.*, 1998; Berneburg *et al.*, 2004). Pang *et al.* (1994) reported that mtDNA⁴⁹⁷⁷ accumulated in sun-exposed skin tissues and also occurred in squamous cell carcinomas (SCC) and precancerous skin tissues. Lately, mtDNA⁴⁹⁷⁷ was

found to reflect the donor's variability rather than chronological ageing (Pang *et al.*, 1994; Koch *et al.*, 2001).

Most recently, the mtDNA³⁸⁹⁵ has been found in skin samples from body sites frequently exposed to solar radiation (Krishnan *et al.*, 2004). Subsequent studies from the same group identified the mtDNA³⁸⁹⁵ which occurred more frequently in usually sun-exposed skin as opposed to occasionally sun-exposed skin (Krishnan *et al.*, 2004; Harbottle and Birch-Machin, 2006). Additionally, multiple sublethal UV irradiations induced the deletion in spontaneously immortalized keratynocytes (HaCaT) (Harbottle and Birch-Machin, 2006). It has been suggested that the frequency of mtDNA³⁸⁹⁵ provides a potential biomarker of cumulative UV exposure in human skin (Harbottle and Birch-Machin, 2006; Harbottle *et al.*, 2010).

1.7 Mitochondrial Nucleoids

Human mtDNA is arranged into DNA-protein structures termed 'nucleoids', which are thought to be the units of genetic inheritance (Jacobs *et al.*, 2000; Wang and Bogenhagen, 2006). The characterization of mtDNA-organization and dynamics is a requirement for understanding the transmission and segregation of wild type and mutant mtDNA between cells and tissues (Lighttowlers *et al.*, 1997; Legros *et al.*, 2004; Chen and Butow, 2005). To date, little is known about how UV-induced mtDNA mutations and deletions affect the nucleoid structure in cells and how the nucleoid structure is affected by mtDNA depletion *per se*.

Nucleoids are likely to have an important role in offering protection against mtDNA damage by bringing together multiple genomes in close proximity enabling DNA repair 18

via gene conversion. Moreover, nucleoids are candidates for the unit of segregation of mtDNA (Jacobs *et al.*, 2000). Nucleoid dynamics is therefore likely to play a key role in determining the level of mutant and wild-type mtDNA in pathological states (Malka *et al.*, 2006; Holt *et al.*, 2007) Apart from a role in the maintenance of mtDNA, little else is known about the physiological role of mitochondrial fusion (Bereiter-Hahn and Voth, 1994; Chan 2006). Indeed, it has been recently reported that mitochondria hyper-fuse and form a highly interconnected network in cells exposed to selective stresses such as UVR (Tondera *et al.*, 2009).

Mitochondrial nucleoids have been described as highly organized structures that tend to regulate their genetic content, containing an associated antioxidant system and mitochondrial transcription control factors. This mitochondrial genetic autonomy may provide a molecular mechanism to explain patterns of mitochondrial genetic inheritance, in addition to facilitating therapeutic methods to eliminate deleterious mtDNA mutations (Gilkerson *et al.*, 2008).

Nucleoid remodeling is supposed to be essential for the maintenance of mitochondrial genome (Kucej *et al.*, 2008). Legros et al. (2004) showed that intermitochondrial fusion and intramitochondrial mobility of endogenous nucleoids and respiratory complexes can ensure functional complementation in normal cells and in heteroplasmic cells containing mutant mtDNA (Legros *et al.*, 2004).

1.8 Mitochondrial Dynamics

By several criteria, mitochondria may be considered dynamic organelles. Firstly, the shape and size of mitochondria are highly variable and controlled by fusion and fission. 19

Secondly, mitochondria are actively transported in cells and they present defined subcellular distribution. Thirdly, the internal structure of mitochondria is not fixed and may change in response to physiological status (Rossignol *et al.*, 2004). Mitochondria dynamics dysfunction has been described as an important causal role in Parkinson Disease (PD) suggesting that drugs modulating mitochondrial function and biogenesis may have important future clinical applications (Mandemakers *et al.*, 2007; Dodson and Guo, 2007).

Ample experimental and clinical data demonstrate that inhibition of either fusion or fission results in deterioration of mitochondrial bioenergetics. On average, each mitochondrion goes through approximately 5 fusion:fission cycles per hour. Measurement of $\Delta\Psi$ m during single fusion and fission events demonstrates that fission may yield uneven daughter mitochondria where the depolarized daughter is less likely to undergo a subsequent fusion event and is more likely to be targeted by autophagy (Legros *et al.*, 2002).

As an intrincate network of dynamic organelles, mitochondrial morphology is a key feature and results from equilibrium between two opposing processes, fusion and fission. Mitochondrial fusion relies on dynamin-related GTPases, the mitofusins (MFN1 and 2) in the outer mitochondrial membrane and OPA1 (Optic Atrophy 1) in the inner mitochondrial membrane (Parrone *et al.*, 2008).

Indeed, it has been recently reported that mitochondria hyper-fuse and form a highly interconnected network in cells exposed to selective stresses. This process precedes mitochondrial fission, triggered by apoptotic stimuli such as UV irradiation or actinomycin D (Tondera *et al.*, 2009).

Preventing mitochondrial fission, by down-regulating expression of Drp1 (Dynaminrelated protein 1) in mammalian cells leads to a loss of mtDNA and a decrease of mitochondrial respiration coupled to an increase in the levels of cellular ROS. Moreover lack of fission leads to mitochondrial dysfunction with concomitant drop in cellular ATP levels, inhibition of cell proliferation and increased autophagy. It has been proposed that mitochondrial fission is required for maintenance of cellular homeostasis (Frieden *et al.*, 2004).

1.9 Mitophagy

Autophagy is a catabolic process that allows for the recycling of cell components under conditions of nutrient depletion and increased energy demand (Mortimore and Poso, 1986; Tasdemir *et al.*, 2007). Autophagy impairment or induction is associated with several diseases, ranging from bacterial and viral infection, to cancer and neurodegenerative conditions (Kirkegaard *et al.*, 2004; Azad *et al.*, 2008; Dagda *et al.*, 2009).

Moreover, autophagy has been recognized as an important mechanism in: cell survival of nutrient deprivation; recycling of damaged mitochondria and anti-tumour drug resilience (Lemaster *et al.*, 1998; Bruno *et al.*, 2007; Ishihara and Mizushima, 2009). More recent reports also suggest that autophagy may have disparate effects on cancer cells: stimulatory or preventative (Vazquez-Martin *et al.*, 2009). Nevertheless, autophagy

and apoptosis evasion is a common tumoural characteristic (Erb *et al.*, 2005; Vazquez-Martin *et al.*, 2009).

Autophagy of the mitochondria (termed mitophagy) is a selective process, targeting organelles with dissipated or low mitochondrial membrane potential (MMP) and activated mitochondrial pore transition (MTP), and it is also recognized as the main degradative pathway involved in mitochondrial turnover (Kim, 2007). These findings imply that increased inorganic phosphate (Pi), ROS, reactive nitrogen species (NOS) and alterations of cellular redox state, which are involved in the MTP, may also promote mitophagy (Rodriguez-Enriquez *et al.*, 2004; Terman *et al.*, 2006; Okamoto *et al.*, 2009). Additionally, ROS formation and MMP alterations have been observed during UVR-induced stress in multiple cells lines and *in vivo* models (Kowaltowski *et al.*, 1996; Aronis *et al.*, 2002; Azad *et al.*, 2008).

Autophagy may maintain the bioenergetic efficiency of cells through continuous targeting and recycling of damaged mitochondria (Twig, 2008). When mitophagy is impaired, rapid accumulation of damaged mitochondria and energy deficiency is observed (Rodríguez-Hernández *et al.*, 2009). Mitophagy may also help remove mitochondria with mtDNA mutations (Kim *et al.*, 2007). Without mitophagy, the retention and build up of dysfunctional mitochondria arising from the intense energy demands of UVR-induced damage repair would present a hazard to accurate damage repair (Lemasters, 2005; Graier *et al.*, 2009).

Although the mechanisms that target mitochondria for mitophagy remain poorly understood, it has been suggested that mitophagy may play a key role in reducing

accumulation of somatic mutations of mtDNA with aging (Lemasters, 2005). Additionally, lysosomes progressively accumulate lipofuscin, further impairing mitochondrial degradation over time (Kurz *et al.*, 2008).

1.10 Aims

The thesis aims are as follows:

- To investigate the effects of sub-lethal SSR doses in cell culture models of tumourigenic and non-tumourigenic skin cells on cell viability, protein content, cellular metabolism and mitochondrial mass
- To clarify the impact of multiple low-doses of SSR in HaCaT cells
- To describe the changes in cellular parameters such as mitochondrial membrane potential, mitochondrial mass and ROS production in response to SSR under diverse cell-density manipulations
- To analyse the occurrence of oxidative damage to nDNA and mtDNA in HaCaT cells post a dose-curve and time-lapse studies
- To quantify the frequency of the solar radiation-associated ΔmtDNA³⁸⁹⁵
- To decipher the importance of energy sources in the mitochondrial morphology adaptations and in the frequency of mitophagy in A375 cells irradiated with low doses of SSR

Chapter 2: Materials and Methods

2.1. Simulated Solar Radiation

The solar irradiator employed in the present study, the Q-sun solar simulator (Q-panel, Cleveland, USA), is designed to provide global solar irradiance typically experienced at noon mid-summer in Florida in the USA i.e. at latitude 25° north of the equator. This is achieved using a Daylight-Q filter (Q-panel) that has a cut-off wavelength of 295 nm and provides an Air Mass (AM) value of 1. An AM value of 1 is defined as when the sun is directly overhead giving, the minimum possible atmospheric attenuation of the extraterrestrial irradiance spectrum, and it is approximated at a latitude 25° north since on the summer solstice (i.e. mid summer, June 21st) the sun is directly above the tropic of Cancer (23.5° north) (Maguire, 2010).

The Q-sun solar simulator is calibrated at a specific level within its sample chamber in the UVA at 340 nm with an irradiance output range from 0.25-0.68 W/m² at 340 nm which adjusts the entire spectrum linearly depending on the electrical current powering the lamp. The Q-sun spectral distribution shown in Figure 2.1 gives a total UV irradiance value of 74 W/m² in the UVA-B between 280 nm and 400 nm, with approximately 72.5 W/m² in the UVA and 1.5 W/m² in the UVB. For the remainder of this thesis the irradiance values and total dose delivered with be given in W/cm² (i.e. J/s/cm²) and J/cm² as these units are commonly used in the biological literature. The Q-sun total UVA-B output would therefore be 83x10⁻⁴ W/cm² therefore delivering 83x10⁻⁴ J/cm² every second and a total dose of 2.5 J/cm² would therefore take 5 minutes. The Q-Sun solar simulator was pre-warmed for 30 minutes before irradiations in order to avoid irradiance and temperature oscillations (as recommended by the manufacturer). Cells were irradiated in pre-warmed phosphate-buffered saline (PBS) (147 mM NaCl, 3 mM NaH₂PO₄, 8 mM KH₂PO₄) to avoid UV absorption by growth medium and the potential phototoxicity of oxidised organic medium components.

Sub-confluent cell cultures were irradiated with SSR with doses ranging from 0.5 up to 7.5 J/cm² (Table 2.1). A SSR of 60 seconds in length corresponds to the energy levels of exposure of 0.5 J/cm². Post irradiations, cells immediately received fresh cell culture media and were incubated for various lengths of time prior to analysis (Details in Table 2.1).

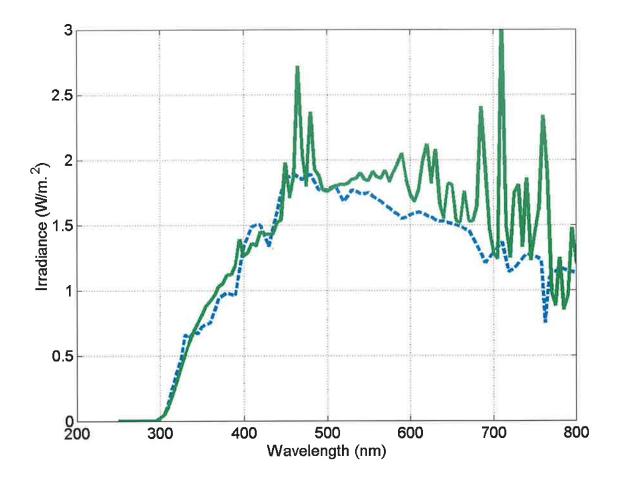


Figure 2.1.- Spectral irradiance for the Q-sun solar simulator operating at 0.68 W/m² at 340 nm (continuous green line) and the typical solar spectrum with an AM value of 1 at a latitude of 25° north (i.e. Florida USA) at solar noon mid-summer (dotted blue line). Data provided by (Q-panel, Cleveland, USA) and confirmed by in house calibration (Maguire, 2010).

2.2. Experimental Design

Experimental Design 1 (Table 2.1) included irradiation lengths of 30, 60 and 300 seconds corresponding to 0.25, 0.5 and 2.5 J/cm² of SSR, respectively.

Experimental Design 2 included cell density manipulations (Low and High Density corresponding to 10.000 and 20.000 cells per well of a 96 wells plate, respectively) as well as dose-curve and time-lapse analysis (Table 2.1).

In the Experimental Design 3 (Table 2.1), cells were exposed to multiple 0.5 J/cm^2 irradiations. A recovering time of 12 hours was allowed between multiple exposures. The final cumulative irradiations doses used for the 1,2,3,4 and 5 times irradiated cells were of 0.5, 1.0, 1.5, 2.0 and 2.5 J/cm², respectively.

For the Experimental Design 4 (Table 2.1), cells were evaluated for dose-response and time-lapse curves responses. The doses associated with 0.5, 1, 5, 10 and 15 minutes of irradiation were 0.25, 0.5, 2.5, 5.0 and 7.5 J/cm², respectively. Time-response studies were performed 0.25, 0.5, 1, 3, 6, 24 and 48 hours post 5.0 J/cm² SSR. The 5.0 J/cm² dose was chosen for these studies as it was observed to cause maximal reduction in mitochondrial mass 3 hours post exposure and was therefore selected to be further investigated over time post exposure.

In Experimental Design 5 (Table 2.1) cells were cultured overnight in complete media and switched to supplemented media for four hours before irradiations. Immediately post irradiation cells were re-loaded with supplemented media used during the overnight cell culturing (to avoid cellular responses due to fresh media addition) and were incubated for a further 4 hours before fluorescence confocal microscopy analysis. Sham irradiated control cultures were handled identically in all Experimental Designs; apart from exposure to SSR (cells were maintained for 60 seconds inside the Q-Sun chamber with the xenon lamp turned off).

Table 2.1: Experimental Design Summary

Experimental Design	Cell Line	SSR Dose (J/cm²)	Time of Analysis	Analysis	
besign		070	(hours)	1	
1 Cellular and Mitochondrial Responses	A375 C32 HaCaT	0 0.25 0.5 2.5	4	Cell Death, DNA quantification, Protein Content, Cellular proliferation, Cellular Metabolism, , Mitochondrial Mass, Time- lapse assessment of mitochondrial mass, Mitochondrial Membrane Potential	
2 Cell Density Manipulation	HaCaT	0 0.25 0.5 2.5 5.0 7.5	0.25 0.5 1 3 6 24 48	Mitochondrial Mass, ROS analysis, Mitochondrial Membrane Potential Analysis	
3 Multiple SSR Exposures	C32 HaCaT	Multiple (1-5 times) 0 0.5	A) 24 48 164 B) 24	 A) Cellular viability, Cellular Metabolism, Protein Content, Mitochondrial Mass B) n/mtDNA quantification, mtDNA deletion analysis 	
4 Dose-Curve and Time-lapse studies	HaCaT	0 0.25 0.5 2.5 7.5	0 0.25 0.5 1 3 6 24 48	n/mtDNA quantification, DNA damage and repair kinetics, mitochondrial ND1 and ND5 genes quantification, ΔmtDNA ³⁸⁹⁵ , mitochondrial mass	
5 Medium Supplementation	A375	0 0.5	4	Cell diameter, lysosomes number, mitochondrial morphology analysis, mitochondria and lysosomes quantification, mitophagy quantification (lysosomes and mitochondria co- localization). 30	

2.3. Cell culture

HaCaT (spontaneously immortalized human keratinocytes) were grown in Dulbbeco's Modified Eagle Medium (DMEM) (Sigma Aldrich, Dorset, UK), supplemented with 10% Foetal Bovine Serum (FBS) (Sigma Aldrich), 2.0 mM L-glutamine (Gibco Biocult, Irvine, Scotland), 100 U/ml Penicillin (Gibco Biocult) and 0.1 µg/ml hydrocortisone (Gibco Biocult)(Table 2.2).

C32 (human amelanotic melanoma cells) obtained from the European Collection of Cell Cultures (ECACC)(UK) were grown in Minimal Essential Mediun Eagle (MEME) (Sigma, Aldrich) which was supplemented with 10% FBS and 1 U/ml penicillin/streptomycin (Gibco)(Table 2.2).

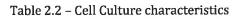
A375 (human malignant melanoma) cells were obtained from the ECACC (UK) and routinely maintained in complete DMEM growth medium containing 10 % foetal bovine serum, 1 U penicillin and 2 mM L-glutamine(Gibco Biocult)(Table 2.2). Cell cultures were maintained in a humidified incubator with 95% humidity and 5% CO₂ at 37°C and culture medium was changed every 48-72 hours.

In Experiment Design 2, HaCaT cells were typically grown to 50-70% before seeding in 96 well plates 24 hours before irradiation / sham irradiation at $1x10^4$ or $2x10^4$ cells per well (equivalent to a 3.2×10^3 cells/cm² and 6.4×10^3 cells/cm²) referred to hence forth as low-density (LD) and high-density (HD) seeded cells respectively.

The cells were seeded in 96 wells plates (NUNC, Roskilde, Denmark) at initial concentrations of 1×10^3 per well and cultured overnight prior to use in subsequent analysis (Experimental Designs 1, 3 and 4)(Table 2.1).

For confocal microscopy analyses (Experimental Design 5), A375 cells were seeded in glass bottom 35mm petri dishes (MatTek Corporation, USA) coated with 0.05 % Type 1 collagen (Gibco) at a density of 30,000 cells per dish and cultured overnight in complete DMEM growth medium.

Cell culture medium was replaced with fresh medium 4 hours before exposure with no further supplements (Control) or supplemented with either 0.6mM glutamine or 6mM glutamine (subsequently referred to as glutamine starvation or supplementation respectively and corresponding to 1/3 and 3 times the 2 mM glutamine as recommended by ECACC), 10 mg/l melanin, 100 mM creatine or 0.5 mM FCCP.



Cell	Cell Type	Main Characteristics	Reference
Line			
A375	Human malignant melanoma	 Cell Growth inhibition by IL1-alpha or IL1- beta at concentrations of 10-30 pg/mL Express TNF-alpha receptors (TNFR55, TNFR75) on their plasma membranes 	 Okada <i>et al.</i>, 1997 Smith <i>et al.</i>, 1994
		 Cell extracts have been shown to contain catalase, 	• Miyamoto <i>et</i> al., 1996
C32	Human amelanotic melanoma	 Resistant to acetylsalicylic acid toxicity Tyrosinase enzyme deficient (enzymatic oxidation of acetylsalicylic acid by tyrosinase to quinine) 	 Vad, et al., 2008 Jimbow et al., 1994
HaCaT	Spontaneously immortalized human keratynocytes	 p53 mutant Expression of MMP-13 (Collagenase-3) Epimorphin inhibition of terminal differentiation induced by calcium influx. 	 Lehman et al., 1993 Johansson et al., 1997 Okugawa and Hirai, 2008

2.4. Pro-oxidant and anti-oxidant treatments

As positive and negative controls for oxidant and antioxidants culturing conditions, HaCaT cells were incubated with 3% H₂O₂ (Sigma Aldrich) for 20 minutes or 1.0 mM Reduced Glutathione (GSH) (Sigma Aldrich) for 60 minutes before further analysis (Experimental Design 2).

2.4. Cellular Response

2.5.1. Detection of Cell Death

A375, C32 and HaCaT cells were harvested 4 hours post irradiation, washed with cold PBS and cell death was measured by flow cytometry using the Annexin V-fluoresceinisothiocyanate (FITC) staining which measures phosphatidylserine exposure. Staining was performed according to the kit manufacturer's instructions (BD Pharmingen). Briefly, cells were recovered by trypsinization, resuspended in 200ul of binding buffer and incubated with 15µl of Annexin V-FITC for 15 min on ice. Finally, 5µl of propidium iodide were added for 5 min and stained cells were examined using a Partec PAS III flow cytometer. A minimum of 10,000 cells were analysed per exposure regime, in triplicate.

2.5.2. DNA quantification

Cellular DNA was measured in A375, C32 and HaCaT cells seeded at a density of 1000 cells/per well in 96 wells plates using PicoGreen dsDNA kit (Molecular Probes, USA), following the manufacturer's instructions. In brief, PicoGreen dye was diluted 1:200

with 1x Tris/EDTA buffer. 100µl of a dye solution was added per well containing the irradiated cells and left to stain for 10 minutes in the cell incubator protected from light. Fluorescence was measure in a fluorescence microplate reader (Tecan GENius) using emission and excitation wavelengths of 520 nm 480 nm, respectively. Data was normalized against sham-irradiated control fluorescence and used to normalise other assays per DNA content per well (From assays 2.3.3. to 2.4.2.). Data are presented as the mean +/- standard deviation of 3 or more separate experiments. Statistical analysis was performed as described in section 2.12.

2.5.3. Protein Quantification

Total protein was determined in cells seeded at a density of 1000 cells/per well using a Bradford Assay reagent (Bio-Rad, UK). The Bradford Protein Assay is a dye binding assay based on the proportional colour change of a dye in response to protein concentration variation (Bradford, 1976). Briefly, cell culture medium was removed and cells were washed 3 times with pre-warmed PBS, 100 µl of Bradford assay solution (6% (v/v in PBS) was added per well and incubated for 15 minutes in room temperature. Bradford dye was then removed, cells were washed 3 times in pre-warmed PBS and phenol red free medium was added to cells. The absorbance was read at 595nm in a microplate reader (Tecan GENius). Data were normalized against relative DNA content and expressed as a percentage of sham-irradiated controls. Data are presented as the mean +/- standard deviation of 3 or more separate experiments. Statistical analysis was performed as described in section 2.12.

2.5.4. Metabolic Activity I - Cell Proliferation Assay

The Alamar Blue assay (Invitrogen) which is reduced to a fluorescent substrate by cellular dehydrogenases, was used to quantify cell proliferation as previously described (Pagliacci *et al.*, 1993). Briefly, cells from each cell line were seeded in a minimum of 6 wells of 96-well plates in 200µl of culture medium. Cells were seeded at 1000 cells/well density on 96 wells plate. For experimental analysis the Alamar Blue was used at a 5% solution on DMEM without Phenol Red. Cells were SSR irradiated, received fresh cell culture medium and were kept in the cell incubator for 4h at 37°C (Experimental Design1) or the plates were further incubated and the assay was performed 1, 4 and 7 days post SSR (Experimental Design 3). Alamar Blue was added in a volume of 100ul per well to the cells and incubated for 30 minutes. The plate was read in a fluorescence microplate reader (Tecan GENius) (excitation, 530 nm; emission, 590 nm). Absorbance values were normalized against relative DNA content and expressed as percentage of sham-irradiated controls. Data are presented as the mean +/- standard deviation of 3 or more separate experiments. Statistical analysis was performed as described in section 2.12.

2.5.5. Metabolic Activity II - Measurement of Mitochondrial Metabolism

The Biolog Redox Dye Mix (Biolog, USA) is a tetrazolium-based reagent that is reduced to a soluble purple formazan product by live cells and it is used as an indicator of cellular metabolism (Slater *et al.*, 1963). The quantity of formazan product is considered proportional to mitochondrial metabolism (Figure 2.2). 1X Biolog Redox Dye Mix was prepared from a 6X concentrate stock solution diluted to the 1X working solution by

diluting it on pre-warmed PBS. Growth medium was removed from the cells cultivated in 96 well plates. Cells were loaded with 100 μ l of 1X Biolog Redox Dye and left in the incubator for 15 minutes. Plates were read using a microplate reader (Tecan GENius) which measured the absorbance of each sample at a wavelength of 590nm. Data were normalized against DNA content and expressed as a percentage of sham-irradiated controls. Data are presented as the mean +/- standard deviation of 3 or more separate experiments. Statistical analysis was performed as described in section 2.12.

2.5.6. Metabolic Activity III - Cell Viability Assay

The relative number of viable cells was determined colorimetrically using the 3-(4,5dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich) assay as previously described (Adhami *et al.*, 2003). Following SSR, the cells received fresh cell culture medium and were incubated at 37^{0} C in 5% CO₂ for 4 hours. For the MTT assay, 20μ l of MTT solution (5mg/ml) was added to each well of a 96-well plate, and incubated for 1h. After this incubation period the medium was discarded, the cells were washed with 100μ l of PBS and 100μ l of DMSO was added to each well to dissolve the formazan crystals formed after conversion of the tetrazolium salt by mitochondrial dehydrogenases. Formazan absorbance was measured at 540nm using a microplate reader (Tecan GENius). Data were normalized against relative DNA content and expressed as a percentage of sham-irradiated controls. Data are presented as the mean +/- standard deviation of 3 or more separate experiments. Statistical analysis was performed as described in section 2.12. Diagramatic representation of the main cellular substrates measured by the mitochondrial metabolism assays is shown in Figure 2.2.

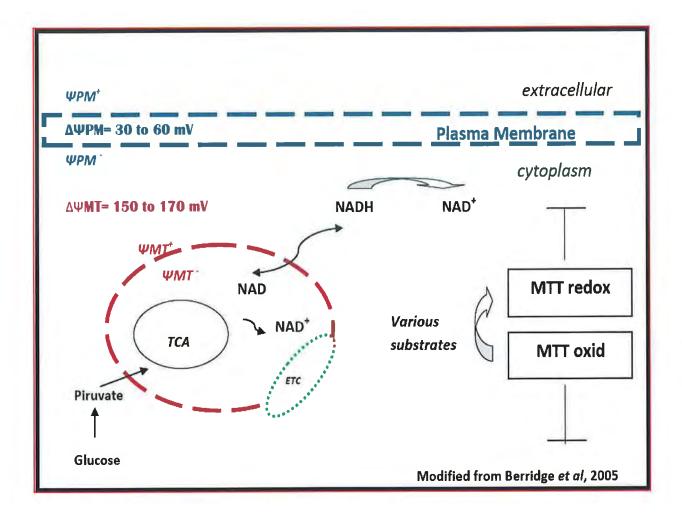


Figure 2.2: Diagram representative of the main cellular substrates and compartments associated with the formazan production from tetrazolium-based salts in assays commonly used to measuring metabolic activity (Alamar Blue, Biolog Redox Dye and MTT assays). (ΨPM – Plasma membrane potential, ΨMT – Mitochondrial Membrane Potential, TCA – Tricarboxilic Acids Cycle, ETC – Electron Transport Chain, NADH – Nicotinamide Adenine Dinucleotide , NAD – Nicotinamide Adenine , MTT - 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide).

2.6. Mitochondrial Analysis

2.6.1. Measurement of Mitochondrial Mass

2.6.1.1. MitoTracker Green

Mitochondrial mass was measured using the fluorescent dye, Mito-Tracker Green FM (Molecular probes). Mitotracker Green accumulates in the mitochondrial matrix where it covalently binds to mitochondrial proteins by reacting with thiol groups of cystein. MitoTracker Green FM accumulates into the mitochondrial matrix independently of the MMP. An increase in green fluorescence indentifies an increase in mitochondrial mass (Keij et al., 2000). In brief, growth medium was removed and cells were rinsed 3 times with pre-warmed and freshly prepared Mg²⁺/Ca²⁺ buffer (130 mM NaCl, 5 mM KCl, 1mM Na₂HPO₄, 1mM MgCl₂ and 1 mM CaCl₂). Cells were loaded with 20 ul of 150nM MitoTracker Green FM dissolved in Mg²⁺/Ca²⁺ buffer, in a 96 well plate and incubated for 20 minutes in the dark at room temperature. Cells were then washed 3 times with Mg²⁺/Ca²⁺ buffer and fluorescence intensity was measured in a fluorescence plate reader (TECAN GENius) using an excitation wavelength of 485nm and emission wavelength of 535nm. MitoTracker Green fluorescence was corrected for background using cell-free Mg²⁺/Ca²⁺ buffer blanks and normalised against relative DNA content before being expressed as a percentage of sham-irradiated control values. Statistical analysis was performed as described in section 2.12.

2.6.1.2 MitoTracker Red

In order to confirm mitochondrial mass variation induced by SSR, A375 and C32 cells were harvested 4 hours post SSR, washed with cold PBS and then loaded with 50 mM of MitoTracker Red (Molecular Probes) in cell culture media for 10 minutes. When used at a low concentration, the MitoTracker Red dye accumulates in the inter-mitochondrial membrane space in a mitochondrial membrane potential dependent manner (Ishihara *et al.*, 2003). To perform a positive control, a cell suspension aliquot was incubated for 20 minutes with 250 nM trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) (Sigma). This depolarization agent was added before the MitoTracker Red staining and flow cytometry analysis. Mitochondrial fluorescence was measured by flow cytometry analysis using a Partec PAS III flow cytometer. A minimum of 10,000 cells were analysed per exposure regime, in triplicate.

2.6.2. Measurement of mitochondrial membrane potential

MMP measurement was performed using Tetramethyl Rhodamine Methyl Ester (TMRM) (Invitrogen). TMRM is a lipophylic cationic dye that binds to inner and outer mitochondrial membranes and accumulates in proportion to MMP (Scaduto and Grotyohann, 1999). A drop in mitochondrial membrane potential is indicated by a decrease in the red signal of TMRM. Cell culture medium was removed, cells were washed in pre-warmed PBS and loaded with 50nM TMRM in phenol red free, serum free cell culture medium and stained for 10 minutes in the incubator.

TMRM dye was removed, cells were washed 3 times with PBS, cells received fresh phenol red free medium and fluorescence measured in a micro plate reader (TECAN GENius) using an excitation and emission wavelengths of 540 and 595 nm respectively. Background correction and normalization by relative cell DNA content was performed for each data set and the results expressed as a percentage of sham-irradiated cells. Experiments were independently performed in triplicate. Error bars represent two standard error of the mean. Statistical analysis was performed as described in section 2.12.

2.6.3. ROS formation assay

Cells were incubated with 2'7'-dichlorofluorescein (H2DCF-DA) (Invitrogen, OR, USA) as previously described (Kushnareva *et al.*, 2002). In brief, 10 μ M H2DCF-DA (diluted in cell culture medium) was added to cells in 96 wells plates for 30 min at 37°C. The dye was then removed and cells were washed twice in PBS. DCF fluorescence was read in a GENios plate reader (Tecan) using an excitation wavelength of 485nm and emission wavelength of 535nm. Three independent experiments were performed and mean H2DCF-DA fluorescence in each sample was expressed as a percentage of non-irradiated controls +/- standard deviation of the mean. Statistical analysis was performed as described in section 2.12.

2.7. DNA analysis

2.7.1. Isolation of DNA

Total DNA was extracted using a DNeasy Tissue Kit (Qiagen, UK). The concentration of total cellular DNA was determined by Picogreen® dsDNA Quantitation Kit (Molecular Probes). DNA purity was verified spectrometrically using a He λ ios spectrophotometer. DNA quality was measured by calculating the absorbance of DNA at wavelength 260nm divided by the DNA absorbance at wavelength 280 nm. DNA samples with ratios ranging from 1.8 and 2.0 were considered for further analysis. All the samples were adjusted to the same concentration ($10\mu g/\mu l$) prior to their use for PCR analysis to remove any variation between samples amplification due to DNA template loading.

2.7.2. Analysis of mitochondrial DNA deletions

Primers were synthesized by Sigma-Aldrich (Germany) (Table 2.3). To detect mtDNA⁴⁹⁷⁷, primers CD1 and CD2, specific to regions flanking the Δ mtDNA⁴⁹⁷⁷ deletion-junction sites were chosen. PCR was carried out under conditions that allowed a product to form only if the deletion has occurred, as previously described (Koch *et al.*, 2001). For the mtDNA³⁸⁹⁵ the primers previously described (Krishnan *et al.*, 2004) were used (Table 2.3). Briefly the PCR primers L404 and H4676 anneal outside the Δ mtDNA³⁸⁹⁵ deletion site.

During DNA amplification the short polymerase extension time (30s) did not permit amplification of wild-type PCR products instead permitting only amplification across deleted mtDNA fragments (Krishnan *et al.*, 2004). A mitochondrial conserved region of mtDNA was amplified as an internal control for the relative mtDNA present in the samples (ConMito). Similarly, a nuclear housekeeping gene region (18S) was selected for amplification as a control for the relative nuclear DNA loading. The primer sequences, genomic region amplified and PCR conditions employed during the aforementioned are shown in Table 2.3. Conventional PCR analysis was performed in a Techne-Touchgene gradient thermal thermocycler. Amplification was conducted in a final volume of 20 μ l containing 1x Reddy Master mix (Sigma) 200 ng DNA and 0.1 μ M of each primer. PCR products were eletrophoresed on a 1% agarose gel in TAE and stained with ethidium bromide. All PCR analyses were performed in triplicate.

Name	Sequence	Product Size	
Δmt	DNA ⁴⁹⁷⁷ Deletion Analysis (CD F/R1) (CD F/R2)		
Forward	GAG AAC CAA CAC CTC TTT ACA GTG A		
Reverse 1 (Mutant)	TAT TCG AGT GCT ATA GGC GCT TGT CAG	271bp	
Reverse 2 (Wild Type)	GGA TAC TAG TAT AAG AGA TCA GGT TCG TC	408bp	
	Human 18S		
H18S Forward	GTA ACC CGT TGA ACC CCA TT	155bp	
H18S Reverse	CCA TCC AAT CGG TAG TAG CG		
	Mitochondria Conserved Region (ConMito)		
ConMito Forward	CTT ACC ACG CTA CTC CTA CC	526bp	
ConMito Reverse	AGG ACT CCA GCT CAT GCG CC		
Δ	mtDNA ³⁸⁹⁵ Analysis by Conventional PCR (a)		
L404	CTT TTG GCG GTA TGC ACT TT	~200bp	
H4676	GAT TAT GGA TGC GGT TGC TT		

Table 2.3: Primers used for conventional PCR analysis of mtDNA and nDNA.

(a) From Krishnan et al., 2004

2.8. Relative mitochondrial genome frequency analysis

Mitochondrial genome copy number was determined using the qRT-PCR as previously described (Wong and Cortopassi, 2008). In brief, qRT-PCR detection and quantification of the nuclear single copy gene of cystic fibrosis (CF) and the mitochondrial Cytochrome b (Cyt b) genes were performed using primer sets and conditions detailed in Table 2.4, to quantitatively assess variation in mtDNA frequency post multiple SSR in HaCaT cells (Experimental Design 4). Additionally, the ND1 and ND5 mitochondrial genes were quantified and normalized to the Cyt b amplification results to assess potential differences in copy numbers in the major (ND5) and minor (ND1) arc regions of mtDNA. qRT-PCR quantification was performed in triplicate, in a total reaction volume of 20µl. The reaction mixture consisted of 5μ l of DNA template (15ng) diluted in MilliQ water, 1X SYBR Green PCR kit (Roche) and 0.2 μ M of forward and reverse primers. qRT-PCR was performed in an LC480 Roche Thermocycler. The specificity of the amplification products was confirmed by high resolution melt analysis and by separation on 1.2% agarose gel. The mtDNA/nDNA ratio was obtained by the relative quantification method analysis mode using the Advanced Relative Quantification LightCycler Data Analysis software (Roche) based on the relative quantification calculations by Pfaffl, 2001 (Pfaffl, 2001).

Table 2.4: Primers used for mtDNA/nDNA and mitochondrial ND1/ND5 genes quantification.

ntitative Real-Time PCR Assay (qRT-I		
	runj – loi dr gree	en)
mtDNA/nDNA quantificatio	on (a)	
AGC AGA GTA CCT GAA ACA GGA A		Cystic Fibrosis
AGC TTA CCC ATA GAG GAA ACA TAA	Nuclear	(Single Copy
		Gene)
GAT TTG GGT ACC ACC CAA GTA TTG		Cytochrome b
AAT AAT CAT GGT GGC TGG CAG TA	Mitochondrial	
ND1/ND5 quantification	(b)	
TCCTCTTCTTAACAACATACC		NADH
GGTGAAGAGTTTTATGGC	Mitochondrial	dehydrogenase 1
AGG CGC TAT CAC CAC TCT GTT CG		NADH
AAC CTG TGA GGA AAG GTA TTC CTG	Mitochondrial	dehydrogenase 5
	AGC AGA GTA CCT GAA ACA GGA A AGC TTA CCC ATA GAG GAA ACA TAA GAT TTG GGT ACC ACC CAA GTA TTG AAT AAT CAT GGT GGC TGG CAG TA ND1/ND5 quantification TCCTCTTCTTAACAACATACC GGTGAAGAGTTTTATGGC AGG CGC TAT CAC CAC TCT GTT CG	AGC AGA GTA CCT GAA ACA GGA AAGC TTA CCC ATA GAG GAA ACA TAANuclearGAT TTG GGT ACC ACC CAA GTA TTGAAT AAT CAT GGT GGC TGG CAG TAMitochondrialTCCTCTTCTTAACAACATACCGGTGAAGAGTTTTATGGCAGG CGC TAT CAC CAC TCT GTT CG

(a) Wong and Cortopassi, 2008

(b) Koch *et al.*, 2001

(c) Genasetti *et al.,* 2007

2.9. Oxidative Mitochondrial DNA Damage and Repair Assay

Oxidative DNA damage and repair were assessed by quantitative PCR (QPCR) as previously described (Santos *et al.*, 2002) (Figure 2.3). The assay is based on the fact that DNA lesions block the progression of any thermostable polymerase on the template when compared to undamaged DNA (Ayala-Torres *et al.*, 2000). A crucial step of QPCR is the concentration of DNA template (Santos *et al.*, 2002). For DNA quantification and subsequent analysis of PCR products, the Picogreen® dsDNA Quantitation Kit (Molecular Probes) was used, following the manufacturer's instructions.

Briefly, total DNA was isolated from control or SSR-irradiated HaCaT cells. DNA was quantified using PicoGreen, and QPCR was performed with equal template DNA. Specific primers were used to amplify a fragment of the DNA polymerase β gene (13.5 kb) to determine nDNA integrity and a large fragment of mtDNA (8.9 kb) to determine mtDNA integrity (Primers and PCR conditions detailed in Table 2.5). For QPCR the Extensor Hi-Fidelity PCR Master Mix (Thermo Scientific) was used. The enzyme has fidelity at least four times higher than standard Taq DNA polymerase. A master mix pre-optimized for amplifications longer that 12Kb was used. To decrease cross-contamination, two different work stations were used for DNA extraction and long PCR product quantification.

After the last QPCR cycle, 10 μ l of PCR product was added to 90 μ l of 1x TE buffer, which was then mixed with 100 μ l of diluted Picogreen reagent (5 μ l of the reagent per ml of 1x TE) in triplicated wells of a 96 wells plate. These solutions were incubated in the dark 47 for 10 minutes and the fluorescence read in a TECAN Genius micro plate reader with excitation and emission wavelengths of 485nm and 530 nm, respectively. The relative PCR amplification was calculated by comparing SSR-irradiated cell data with shamirradiated control data.

The PCR amplification values measured by Picogreen fluorescence were used to mathematically estimate the number of lesions present in DNA, assuming a Poisson distribution (Ayala-Torres *et al.*, 2000; Santos *et al.*, 2002; Santos *et al.*, 2006). Briefly, the amplification of damaged samples (A_D) was normalized to the amplification of a non-damaged control (A_0) resulting in a relative amplification ratio.

These results were then used to determine the lesion frequency per DNA fragment, such that the lesion/strand (average for both strands) in a cell population could be calculated using the formula: $D = - \ln (A_D/A_C)$. (A_D - fluorescence from SSR irradiated cells; A_C - fluorescence of sham-irradiated controls).

Statistical analysis was performed using the Student's *t*-test and all the samples were compared to sham-irradiated controls. All the values are expressed as mean +_S.E.M.

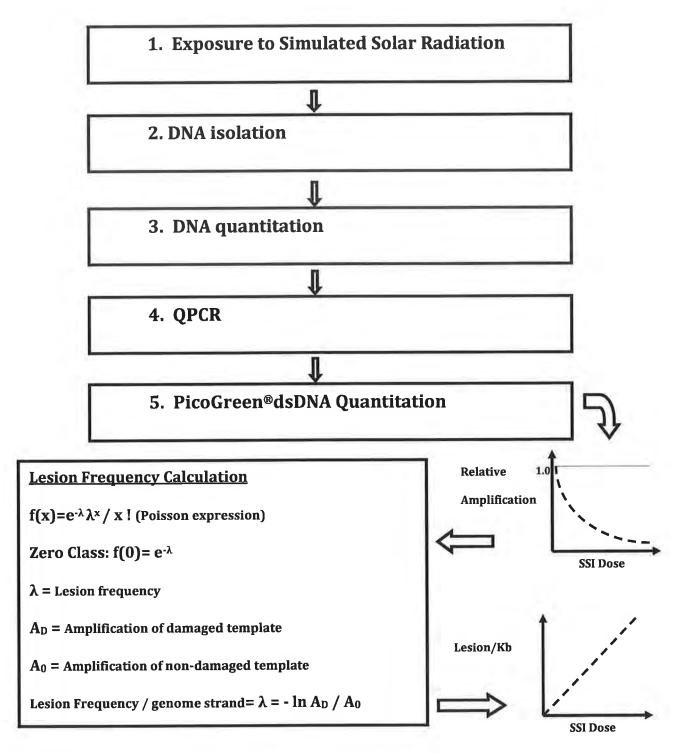
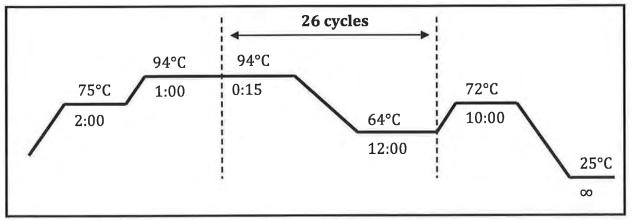


Figure 2.3: Schematic representation of the steps necessary for the oxidative mtDNA damage and repair using the gene-specific QPCR assay (Modified from Santos *et al.*, 2006).

Table 2.5: Primers used for mtDNA damage and repair analysis.

Gene	Sequence	Position	Amplicon Size	
	Nuclear Genes			
hprt -F	TGG GAT TAC ACG TGT GAA CCA ACC	14577		
hprt-R	GCT CTA CCC TCT CCT CTA CCG TCC	24997	10.4 Kb	
DNA pol. β-F	CAT GTC ACC ACT GGA CTC TGC AC	2372		
DNA pol. β-R	CCT GGA GTA GGA ACA AAA ATT GCT G	3927	12.2 Kb	
Mitochondrial Genes				
Mito-F	TCT AAG CCT CCT TAT TCG AGC CGA	5999	8.9 Kb	
Mito-R	TTT CAT CAT GCG GAG ATG TTG GAT GG	14841		

PCR Conditions



(a) Santos *et al.*, 2006.

2.10. Quantification of mtDNA³⁸⁹⁵

mtDNA³⁸⁹⁵ analysis was performed as previously described (Harbottle and Birch-Machin, 2006) using primers specific to the regions flanking the mtDNA³⁸⁹⁵ deletion site and a Taqman probe with homology to the deletion-junction site. Briefly, the method uses an internal standard probe (IS-probe – Table 2.6) in the cytochrome b region of the genome (Rogounovitch et al., 2002), to estimate the copy number of mtDNA. The frequency of mtDNA³⁸⁹⁵ is determined by a probe (3895-probe – Table 2.6) which spans the break point of the deletion ensuring that the fluorescence emission is proportional to the deleted mtDNA present in the sample. Quantification of the level of deletion is determined by comparison of the ratio of the internal standard to the Δ mtDNA³⁸⁹⁵. The primer sequences, genomic region amplified and PCR conditions referent to the above mentioned amplifications are shown in Table 2.6. Figure 2.4 illustrates primer and probe localization on the mitochondrial genome. qRT-PCR was performed on a LC480 Roche Thermocycler using a QuantiTect Probe PCR Kits (Qiagen) following the manufacturer's instructions. All qRT-PCR analyses were performed in triplicate and mtDNA³⁸⁹⁵ frequency was normalized against the IS reference gene and expressed as a ratio to sham-irradiated control mtDNA³⁸⁹⁵ quantification. Sham-irradiated control cells are expected to have a basal level of Δ mtDNA³⁸⁹⁵.

Table 2.6: Primers used for $\Delta mtDNA^{3895}$ quantification

Name	Sequence	Gene	
ΔmtDNA3895 qRT-PCR (Taqman Probes)			
ISF	GAT TTG GGT ACC ACC CAA GTA TTG	Cytochrome b	
ISR	AAT AAT CAT GGT GGC TGG CAG TA		
IS-Probe (b)	CAC CCA TCA ACA ACC GCT ATG TAT	Dye: Hex	
	TTC GTA CA		
3895F	CAA CCC TCG CCC ATC CTA	3895 deletion	
3895R	CCT GCA AAG ATG GTA GAG TAG	junction	
	ATG AC		
3895-Probe (a)	TGC TAA CCC CAT ACC CCG AAA ATG	Dye: Fam	
	TTG G		

(a) Koch *et al.*, 2001

(b) Harbottle and Birch-Machin, 2006

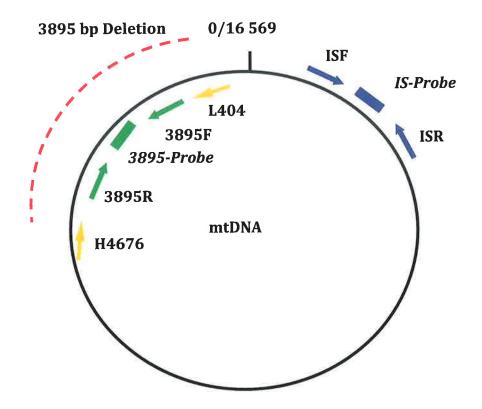


Figure 2.4: Illustration of the primers and primer site locations employed in mtDNA³⁸⁹⁵ analysis. PCR primers and Taqman probes are represented. Primers ISF/ISR and IS-probe anneal to both wild-type and deleted mtDNA. Detection of the mtDNA³⁸⁹⁵ was performed with primers 3895F/3895R and 3895-Probe which anneals to the 3895bp deletion junction. Primers L4040 and H4676 flank the deletion region and generate an amplicon only from deleted genomes under the restrictive PCR conditions employed. mtDNA³⁸⁹⁵ deleted genes include 12s rRNA, 16s rRNA, ND1 and the promoter for the transcription of both the H and L strands (from Harbottle and Birch-Machin, 2006).

2.11. Mitophagy Analysis

LysoTracker Green (LTG) (Molecular Probes) was added to growth medium to a final concentration of 50 nM and cells were incubated for 20 minutes. MitoTracker Red (MTR) was added to a final concentration of 50 nM and cells incubated for 5 minutes. Cells co-loaded with both fluorescent dyes were then washed in PBD, covered with phenol red-free DMEM and immediately analyzed by confocal microscopy.

An LSM 510 Confocal Laser Scanning Inverted Microscope (Carl Zeiss) equipped with a helium neon laser (excitation wavelength 543 nm) and an argon ion laser (excitation wavelength 488 nm) was used. The objective used was an oil immersion plan-neofluar 63x/1.4. The red fluorescence of MTR (excitation 579 nm and emission 599 nm) and the green fluorescence of LTG (excitation 504 nm and emission 511 nm) were measured immediately to avoid dye leakage. Laser excitation energy was set to 0.5% to minimize photo-bleaching and photo-damage.

Mitophagy events were predicted using the Image Processing Kit 4.0.25 software (Carl Zeiss) analysis of LTG / MTR fluorescence co-localization. Data acquisition was optimized and kept constant for all subsequent data acquisition. The fluorescence of control cell populations was set as a base-line to enable semi-quantitative comparative analysis.

Cell area and organellar fluorescence data were acquired from at least 50 cells per experimental condition. Each individual cell's area, and superimposed acidic organelle fluorescence was identified as a region of interest (ROI). Mitochondrial and lysosomal fluorescence were acquired for each individual cell selected as a region of interest (ROI) using the Image Processing Kit 4.0.25 software. Mitophagy events were quantified as the selected ROI of co-localization fluorescence (yellow fluorescence) from 7 z-stack superimposed images for each individual cell.

2.12. Statistical analysis

Data are presented as the mean of 3 or more experiments \pm standard error of the mean. Differences between treated and control cell populations were analyzed by either the student's *t* test or a one-way analysis of variance (ANOVA), using p < 0.05 as the criterion for significance - (*) represents p<0.05 and (**) p<0.01 are represented(*) and (**), respectively.

Chapter 3: Results

3.1. Cellular Response

3.1.1. Detection of Cell Death

SSR induced a decrease in apoptosis in A375 cells (3.5 % in 2.5 J/cm² irradiated cells v 4.1 % in sham-irradiated controls (Figure 3.1). A reduction in necrotic cells was observed post SSR in C32 cells (20.3% in the 1.0 J/cm² SSR irradiated cells v 27% in sham-irradiated control) (Figure 3.2). It was observed that necrotic cell number increases linearly with SSR dose in HaCaT cells peaking in cells irradiated with 2.5 J/cm² (Figure 3.3). Representative flow cytometric analysis of A375 cells using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen[™]) is presented in Figure 3.4.

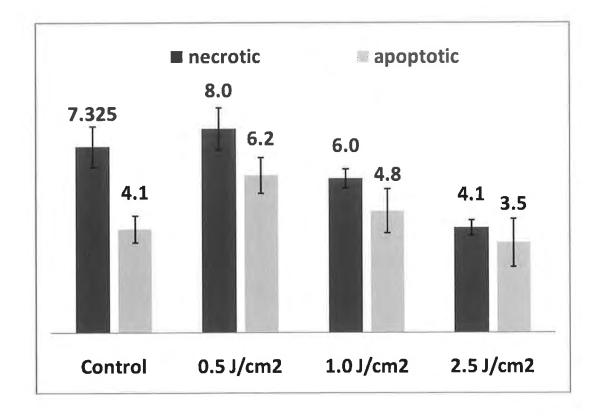


Figure 3.1: Evaluation of necrosis and apoptosis by phospatidylserine exposure and PI staining in A375 cells post SSR. Flow cytometric analysis of A375 cells was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen[™]). A375 cells were irradiated with SSR doses ranging from 0 to 2.5 J/cm² and analysed by flow cytometry 4 hours post irradiation. Black bars – percentage of necrotic cells. Grey Bars – percentage of apoptotic cells. Results are expressed as a percentage of total cell number analysed (minimum of 10.000 cells in triplicate).

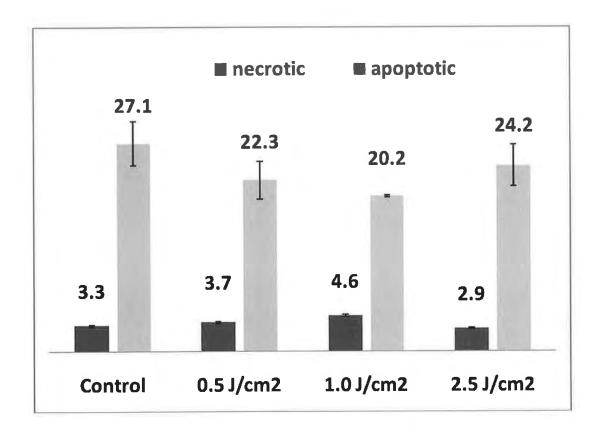


Figure 3.2: Evaluation of necrosis and apoptosis by phospatidylserine exposure and PI staining in C32 cells post SSR. Flow cytometric analysis of C32 cells was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen[™]). C32 cells were irradiated with SSR doses ranging from 0 to 2.5 J/cm² and analysed by flow cytometry 4 hours post irradiation. Black bars – percentage of necrotic cells. Grey Bars – percentage of apoptotic cells. Results are expressed as a percentage of total cell number analysed (minimum of 10.000 cells in triplicate).

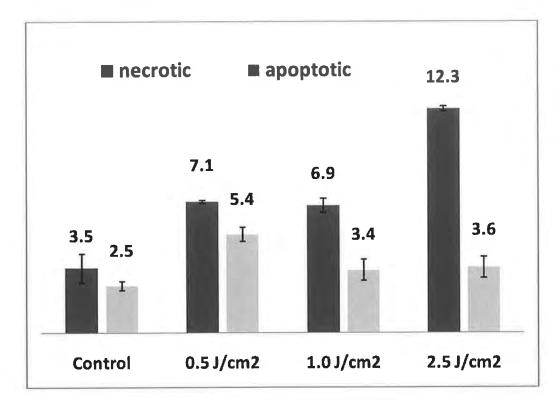


Figure 3.3: Evaluation of necrosis and apoptosis by phospatidylserine exposure and PI staining in HaCaT cells post SSR. Flow cytometric analysis of HaCat cells was performed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen[™]). HaCat cells were irradiated with SSR doses ranging from 0 to 2.5 J/cm² and analysed by flow cytometry 4 hours post irradiation. Black bars – percentage of necrotic cells. Grey Bars – percentage of apoptotic cells. Results are expressed as a percentage of total cell number analysed (minimum of 10.000 cells in triplicate).

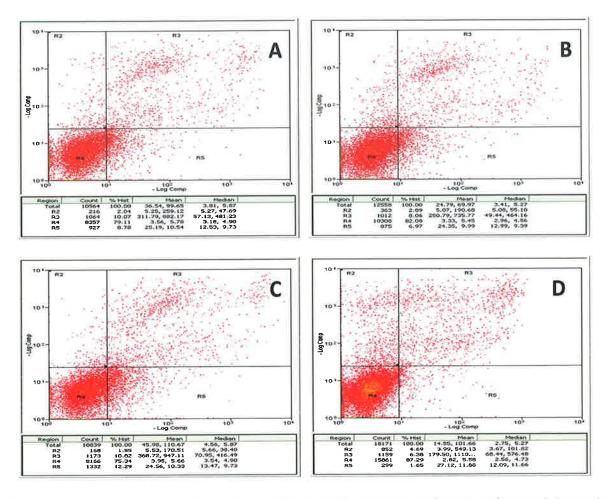


Figure 3.4: Representative flow cytometric analysis of A375 cells using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen[™]). A375 cells were (A) Sham-SSR irradiated or irradiated with (B) 0.5 J/cm², (C) 1.0 J/cm² or (D) 2.5 J/cm². Cells were recovered by trypsinization 4 hours post irradiations and immediately analysed by FITC Annexin V double staining. R2 – Annexin stained cells (early apoptotic cells); R3 – Annexin V + FITC stained cells (apoptotic cells); R4 – Annexin V/ FITC negatives (live cells); R5 – FITC stained cells (necrotic cells). X axis – FITC fluorescence. Y axis – Propidium Iodide fluorescence.

3.1.2. DNA quantification

Total DNA was analysed using the fluorescent PicoGreen assay. Relative DNA content was expressed as a percentage of sham-irradiated controls (Figure 3.5). A modest but significant increase in cellular DNA content was observed in A375 and HaCaT cells 4h post 0.5 J/cm² and in A375 cells exposed to 2.5 J/cm² of SSR (Figure 3.5). In C32 cells, however, a significant reduction in total DNA content was observed for all the SSR studied (Figure 3.5).

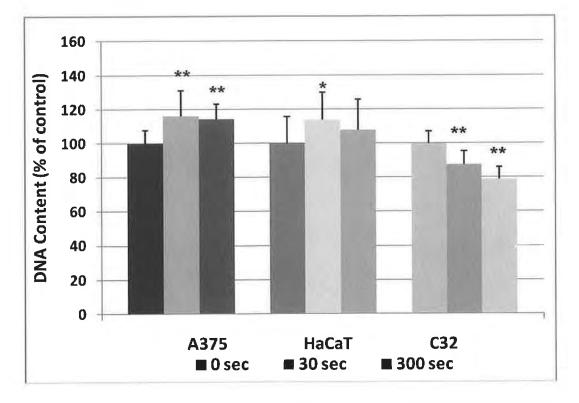


Figure 3.5: Total DNA was determined in A375, C32 and HaCat cells 4 hours post SSR with 0, 0.5 and 2.5 J/cm² (corresponding to 0, 30 and 60 seconds length of exposure) in PBS. Total cellular DNA content was measured using the PicoGreen dsDNA kit in cells seeded in 96 well plates and read in a microplate reader (TECAN Genius). All experiments were performed in triplicate and results are expressed as the mean +/- standard deviation of the mean. Statistical significance was determined by the student's t test and * denotes p<0.05 and ** denotes p<0.01.

3.1.3. Protein Quantification

Total protein was analysed using absorbance analysis of Bradford reagent and normalized against DNA content. Significant changes in protein content were only observed in C32 cells (25 and 45%) 4h post 0.5 and 2.5 J/cm² irradiations, respectively (Figure 3.6).

3.1.4. Metabolic Activity I - Cell Proliferation Assay

To confirm that the chosen SSR doses used were not lethal to any cell line studied, metabolic activity studies were performed using the Alamar Blue assay. It was observed that SSR induced a moderate but significant cell viability reduction in A375 and HaCaT cells exposed to 0.5 J/cm² of SSR, 4 hours post irradiation (Figure 3.7) and that in C32 cells, SSR induced 20% and 60% increase in cell viability measured in the 0.5 and 2.5 J/cm² irradiated cells, respectively (Figure 3.7).

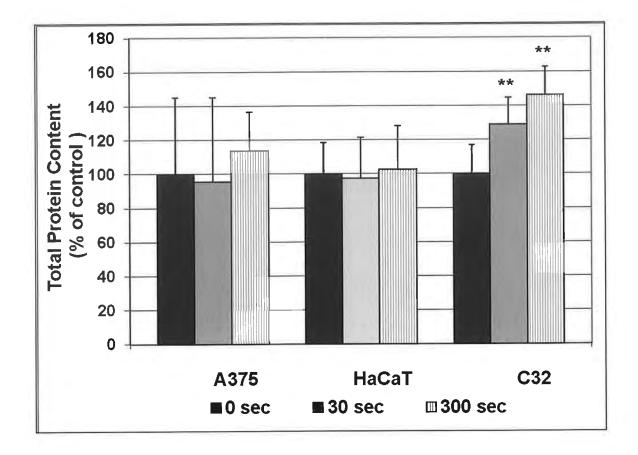


Figure 3.6: Total protein was determined in A375, C32 and HaCaT cells 4 hours post SSR with 0, 0.5 and 2.5 J/cm² (corresponding to 0, 30 and 60 seconds length of exposure) in PBS. Total protein content was measured using the Bradford Assay kit in cells seeded in 96 well plates and read in a microplate reader (TECAN Genius). Protein content was normalized against total DNA content, determined previously, before expressing results as a percent of sham-irradiated control cells (% of control). All experiments were performed in triplicate and results are expressed as the mean +/- standard deviation of the mean. Statistical significance was determined by the student's t test and * denotes p<0.05 and ** denotes p<0.01.

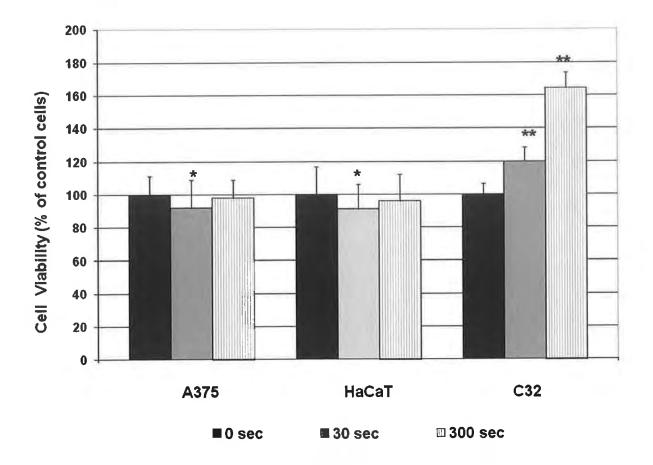


Figure 3.7: Cell proliferation (%) was determined in A375, C32 and HaCaT cells 4 hours post SSR with 0, 0.5 and 2.5 J/cm² (corresponding to 0, 30 and 60 seconds length of exposure) in PBS. Cell proliferation was measured using the Alamar Blue kit in cells seeded in 96 well plates and read in a microplate reader (TECAN Genius). Cell viability was normalized against total DNA content, determined previously, before expressing results as a percent of sham-irradiated control cells (% of control). All experiments were performed in triplicate and results are expressed as the mean +/- standard deviation of the mean. Statistical significance was determined by the student's t test and * denotes p<0.05 and ** denotes p<0.01.

3.1.5. Metabolic Activity II - Measurement of Mitochondrial Metabolism

Relative mitochondrial metabolism was determined in A375, C32 and HaCaT post SSR using the Biolog Redox Dye. A moderate but significant decrease in mitochondrial metabolism was observed in A375 cells exposed to 0.5 J/cm² of SSR whereas a significant increase in the same parameter was observed in C32 cells irradiated with 2.5 J/cm² of SSR (Figure 3.8).

3.1.6. Metabolic Activity III - Cell Viability Assay

The MTT assay results obtained were expressed as a percentage (%) of sham-irradiated controls. The results showed that cellular metabolism post SSR was significantly increased only in C32 cells (Figure 3.9).

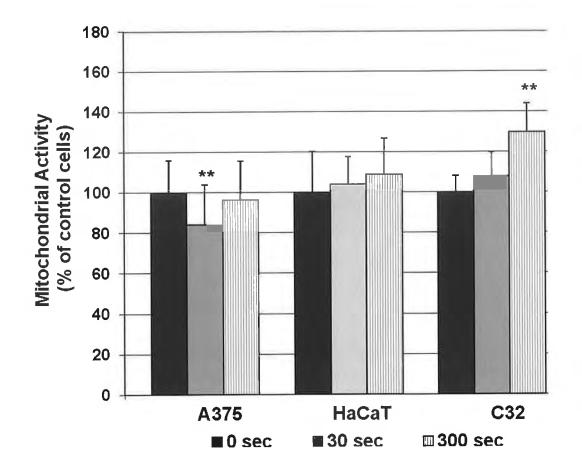


Figure 3.8: Metabolic activity II - Mitochondrial Metabolism was determined in A375, C32 and HaCaT cells 4 hours post SSR with 0, 0.5 and 2.5 J/cm² (corresponding to 0, 30 and 60 seconds length of exposure) in PBS. Mitochondrial metabolism was measured using the Biolog Redox Assay in cells seeded in 96 well plates and read in a microplate reader (TECAN Genius). Metabolic activity II was normalized against total DNA content, determined previously, before expressing results as a percent of sham-irradiated cells (% of control). All experiments were performed in triplicate and results are expressed as the mean +/- standard deviation of the mean. Statistical significance was determined by the student's t test and * denotes p<0.05 and ** denotes p<0.01.

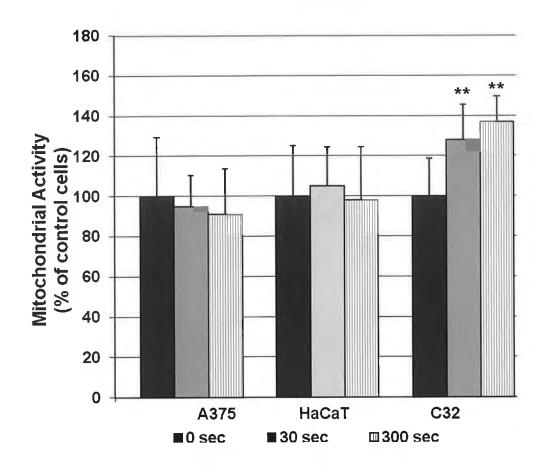


Figure 3.9: Metabolic Activity III - Cellular Metabolism. Cellular Metabolism was determined in A375, C32 and HaCaT cells 4 hours post SSR with 0, 0.5 and 2.5 J/cm² (corresponding to 0, 30 and 60 seconds length of exposure) in PBS. Cellular metabolism was measured using the MTT colorimetric assay in cells seeded in 96 well plates and read in a microplate reader (TECAN Genius). Cell Metabolism was normalized against total DNA content, determined previously, before expressing results as a percent of sham-irradiated cells (% of control). All experiments were performed in triplicate and results are expressed as the mean +/- standard deviation of the mean. Statistical significance was determined by the student's t test and * denotes p<0.05 and ** denotes p<0.01.

3.1.7. Mitochondrial Analysis

3.1.7.1. Measurement of Mitochondrial Mass

3.1.7.1.1. MitoTracker Green

Relative changes in mitochondrial mass in A375, C32 and HaCaT cells were determined using MitoTacker Green FM. A significant decrease in mitochondrial mass was observed in A375 and HaCaT cells exposed to 0.5 J/cm² SSR and in A375 cells exposed to 2.5 J/cm², 4 hours post irradiation (Figure 3.10). In C32 cells, however, SSR induced a significant increase in mitochondrial mass 4h post all irradiation doses dose included in this study (Figure 3.10).

In order to elucidate the time-course of mitochondrial mass variation post SSR, A375 and C32 cells were analysed from 2h to 72h post irradiation. The SSR doses used were 0.5 and 1.0 J/cm². In A375 cells, maximal and minimal mitochondrial masses were observed 96h post in 0.5 J/cm² and 1.0 J/cm² irradiated cells, respectively (Figures 3.11). C32 cells presented maximal increase in mitochondrial mass 96h post 0.5 J/cm² SSR and at 20h post 1.0 J/cm² SSR. Maximal decrease in mitochondrial mass was observed in C32 cells 96h post 1.0 J/cm² irradiation (Figure 3.12).

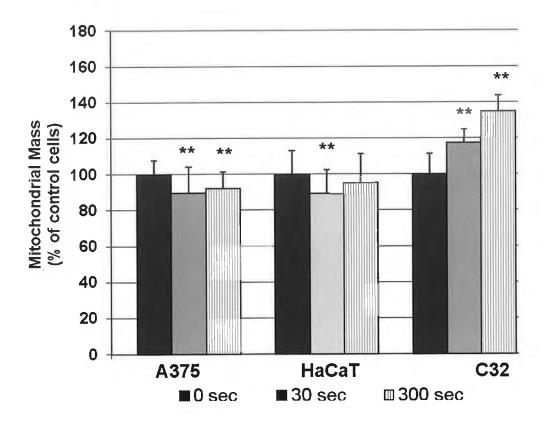


Figure 3.10: Mitochondrial mass was determined in A375, C32 and HaCaT cells 4 hours post SSR of 0, 0.5 and 2.5 J/cm² (corresponding to 0, 30 and 300 seconds length of exposure) in PBS. Mitochondrial mass was measured using the MitoTracker Green Dye in cells seeded in 96 well plates and read in a microplate reader (TECAN Genius). Mitochondrial Mass was normalized against total DNA content, determined previously, before expressing results as a percent of sham-irradiated cells (% of control). All experiments were performed in triplicate and results are expressed as the mean +/- standard deviation of the mean. Statistical significance was determined by the student's t test and * denotes p<0.05 and ** denotes p<0.01

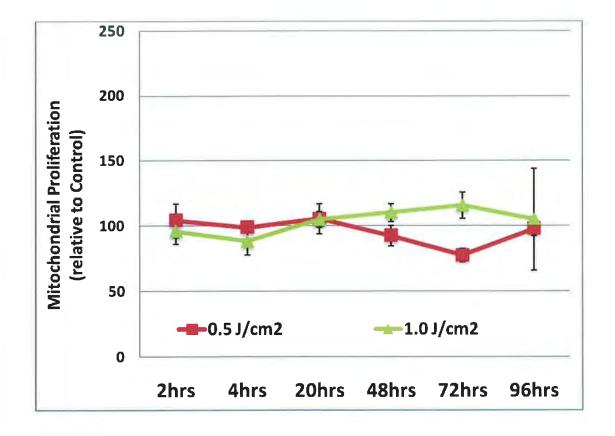


Figure 3.11: Time-course of Mitochondrial Proliferation post SSR in A375 cells. Relative mitochondrial proliferation was determined in A375 cells from 2h to 96h post 0.5 J/cm² and 1.0 J/cm² irradiation using MitoTracker Green Dye fluorescence measured in a micro plate reader. Mitochondrial proliferation is expressed as a percentage of time-matched sham-irradiated controls. Each data point is the mean of 3 replicates and errors are the standard error of the mean.

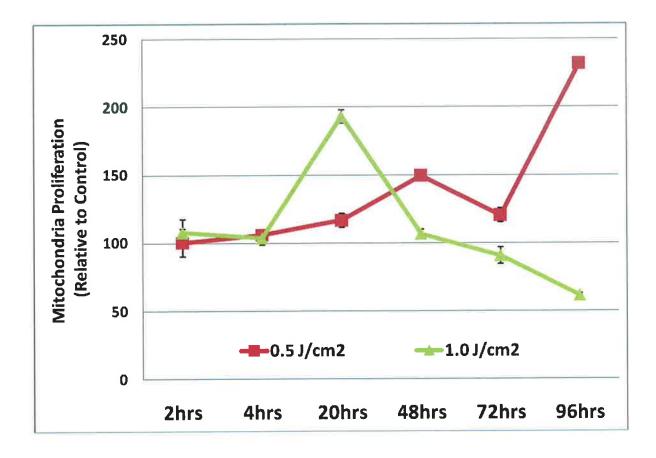
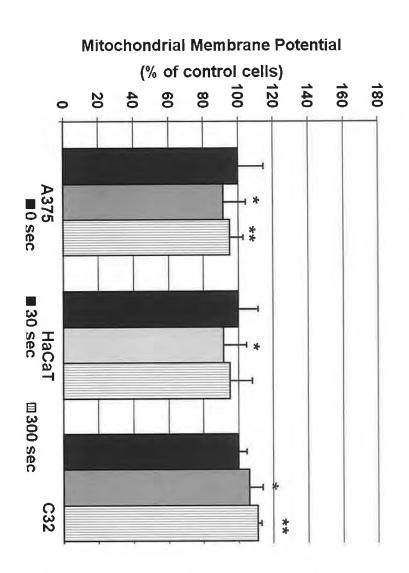


Figure 3.12: Time-course of Mitochondrial Proliferation post SSR in C32 cells. Relative mitochondrial proliferation was determined in C32 cells from 2h to 96h post 0.5 J/cm² and 1.0 J/cm² irradiation using MitoTracker Green Dye fluorescence measured in a micro plate reader. Mitochondrial proliferation is expressed as a percentage of time-matched sham-irradiated controls. Each data point is the mean of 3 replicates and errors are the standard error of the mean.

3.1.7.2. Measurement of mitochondrial membrane potential

Mitochondrial membrane depolarization by SSR was assessed by determining TMRM dye accumulation in the mitochondrial inter-membrane space. MMP analysis of A375 cells demonstrated that a significant mitochondrial depolarization was induced by 0.5 J/cm² and 2.5 J/cm² irradiation doses. In HaCaT cells, only the lower SSR dose (0.5 J/cm²) induced significant mitochondrial depolarization whereas for C32 cells a significant mitochondrial hyper-polarization was observed for both SSR doses evaluated (Figure 3.14).



performed in triplicate and results are expressed as the mean +/- standard deviation of the 96 well plates and read in a microplate reader (TECAN Genius). Mitochondrial Membrane exposure). Mitochondrial Membrane Potential was measured using TMRM Dye in cells seeded in hours post SSR of 0, 0.5 and 2.5 J/cm² (corresponding to 0, 30 and 300 seconds length of mean. Statistical significance was determined by the student's t test and * denotes p<0.05 and **expressing results as a percent of sham-irradiated cells (% of control). All experiments were Potential data was normalized against total DNA content, Figure 3.14: Mitochondrial Membrane Potential was determined in A375, C32 and HaCaT cells 4 denotes p<0.01. determined previously, before

3.1.8. Cell Density and SSR response

3.1.8.1. Mitochondrial mass analysis

 H_2O_2 induced a significant decrease in the mitochondrial mass of HaCaT cells (40% of control), whereas a significant increase (50% of control) in mitochondrial mass was observed post antioxidant treatment (Figure 3.15A). SSR induced an increase in mitochondrial mass for all SSR doses analysed 3 hours post irradiation except in 5.0 J/cm² irradiated cells, where a significant decrease in mitochondrial mass was observed (Figure 3.15A). Changes in mitochondrial mass with time post exposure to 5.0 J/cm² were observed to fluctuate above and below the control at 1, 3 and 6 hours but were less than the control (85-90%) 48 hours later as observed in Figure 3.16A.

3.1.8.2. ROS analysis

Neither H_2O_2 nor GSH altered ROS level in LD seeded cells whereas a significant decrease in ROS was observed in HD cells incubated with both H_2O_2 and GSH (Figure 3.16). No significant change in ROS was observed in the LD cells 3 hours post any SSR dose studied (Figure 3.16B). A significant decrease in ROS was observed in HD cells, post all SSR doses employed except the highest dose (7.5 J/cm²) where levels were comparable to control. In the time course study (Figure. 3.16B), ROS formation was significantly decreased up to 6 hours post exposure in LD and HD cells, except for a modest increase observed in LD cells at 3 hours. ROS levels were observed to fluctuate to levels below, above and below control from 6 to 24 to 48 hours post exposure respectively, except for one data set for HD cells 48 hours post exposure (Figure 3.16B).

3.1.8.3. Mitochondrial membrane potential analysis

 H_2O_2 and GSH induced significant loss of MMP in sham-irradiated HaCaT cells to approximately 15% and 70% of control, respectively (Figure 3.15C) and the amplitude of the effect was cell density dependent. MMP was significantly decreased in LD cells post 1.0, 2.5 and 7.5 J/cm² and was significantly decreased in HD cells post 2.5 and 7.5 J/cm² only (Figure 3.15C). MMP was observed to be significantly less than control in LD and HD cells from 0.25 to 6 hours post exposure to 5.0 J/cm² except for 1 data point in HD cells 0.25 hours post exposure (Figure 3.16C). A significant MMP increase was observed 24 hours post exposure in both LD and HD cells, though was more pronounced in LD cells. MMP was observed to return to control levels or close to control levels by 48 hours post exposure.

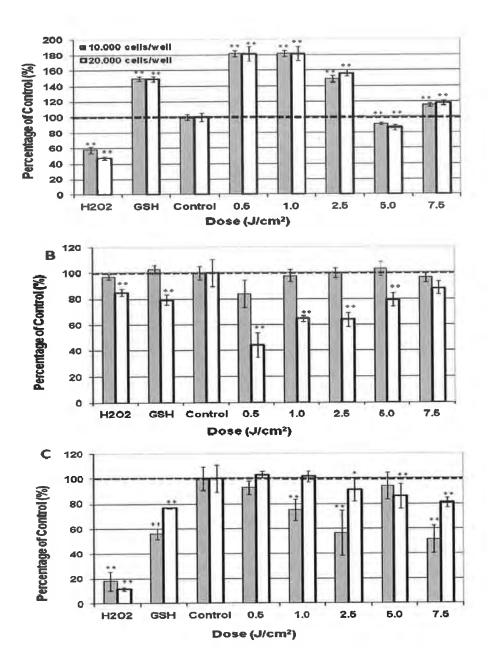


Figure 3.15: The effect of SSR on the (A) mitochondria mass, (B) ROS and (C) MMP observed in \mathbb{R} – 10.000 cells/per well (Low density -LD) or \Box – 20.000 cells per well of 96 well plate (High density – HD). Increasing irradiation doses from 0.25 J/cm² up to 7.5 J/cm² effects on HaCaT cells were analyzed 3 hours post irradiation. MitoTracker Green, DCF and TMRM were used to measure mitochondrial mass, ROS and MMP, respectively. In each case, mitochondrial mass, ROS or MMP are expressed as percentage of sham irradiated (control) samples. Data represent the mean of three experiments,±SEM. Statistically significant difference from sham irradiated cells (Students' *t*-test) are represented (**p*<0.05, ***p*<0.01).

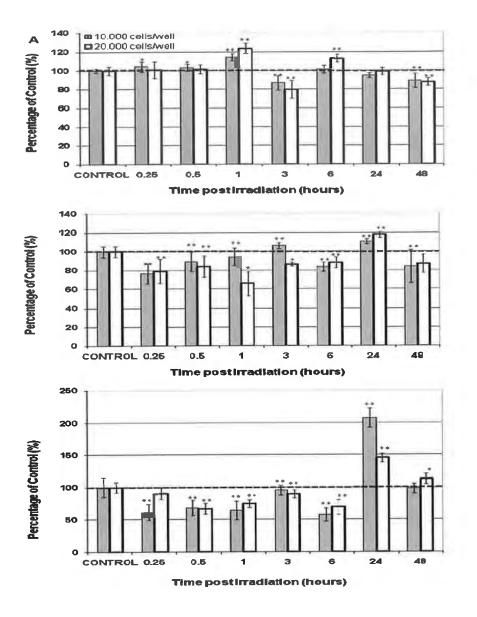


Figure 3.16: HaCaT cells were exposed to a simulated solar radiation dose of 5.0 J/cm² and analyzed 0.25-48 hours later for (A) mitochondrial mass, (B) ROS and (C) MMP. Cells were seeded at either \cong 10,000 cells/per well (Low density –LD) or \square 20,000 cells per well (High density – HD). Sham exposed cell populations were also treated with either hydrogen peroxide (H₂O₂) or reduced glutathione (GSH) and analysed 24 hours later. Results are expressed as a percentage of sham irradiated (control) samples. Data represent the mean of three experiments ± SEM. Statistically significant difference from sham irradiated cells (Students t-test) are denoted by * for p<0.05 and **for p<0.01)

3.1.9. Multiple Simulated Solar Radiations

3.1.9.1. Cellular Viability

A significant increase in cell viability (40% of control) was observed in HaCaT cells 4 days post 1.0 J/cm² of SSR (Figure 3.17A). A single 0.5 J/cm² irradiation did not induce significant change in HaCaT cell viability. 4 days post 2.5 J/cm² irradiation dose a decrease of at least 50% in cell viability was observed. Except for 2.5 J/cm² irradiated cells, a cellular viability comparable to control was observed 7 days post all SSR doses studied suggesting that, under the present study conditions, SSR-induced change in HaCaT cell viability was transient.

3.1.9.2. Protein Content

A consistent 20% increase in protein content was observed in the 1.5 J/cm² irradiated cells 1 day post irradiation and in the 0.5 and 2.0 J/cm² HaCaT cells, 4 days post SSR. The more pronounced changes in protein content in HaCaT cells were observed as a 40% decrease observed 7 days post irradiation for all the SSR doses included in the present study (Figure 3.17B).

3.1.9.3. Metabolic Activity

Significant metabolic activity reduction (20%) was observed in HaCaT cells 4 days post 2.0 J/cm² SSR. The higher dose (2.5 J/cm²) irradiated cells however, presented a 20% increase in metabolic activity, 4 days post irradiation. Cellular metabolism recovered up to control levels 7 days post irradiation, except post higher doses (2.0 and 2.5 J/cm²), when a significant increase (20 and 50%, respectively) in metabolic activity was observed (Figure 3.17C).

3.1.9.4. Mitochondrial Mass

MitoTracker green dye uptake was used as a marker for mitochondrial mass changes post irradiation and it was significantly decreased (approximately 40%) in HaCaT cells 4 days post irradiations, irrespective of SSR dose. A significant increase in mitochondrial mass (70%) was observed in HaCaT cells 7 days post the higher dose (2.5 J/cm²) SSR (Figure 3.17D).

3.1.9.5. Mitochondrial Mass and Metabolic Activity Association

To investigate whether metabolic activity was directly associated with up-regulation in mitochondrial mass proliferation induced by multiple SSR, the Biolog Redox absorbance was normalized against total Mitotracker Green fluorescence, providing relative units of metabolic activity per mitochondrial mass. The more pronounced increase in metabolic activity per mitochondrial mass was observed 1 day post 1.0 J/cm² irradiation (Figure 3.19A). Multiple SSR induced an approximately 20% decrease in metabolic activity per mitochondrial mass studied except for the higher dose included in the present study (cumulative 2.5 J/cm²) in which 60% decrease was observed 4 days later followed by a 20% increase in the variable ratio, 7 days later (Figure 3.19A).

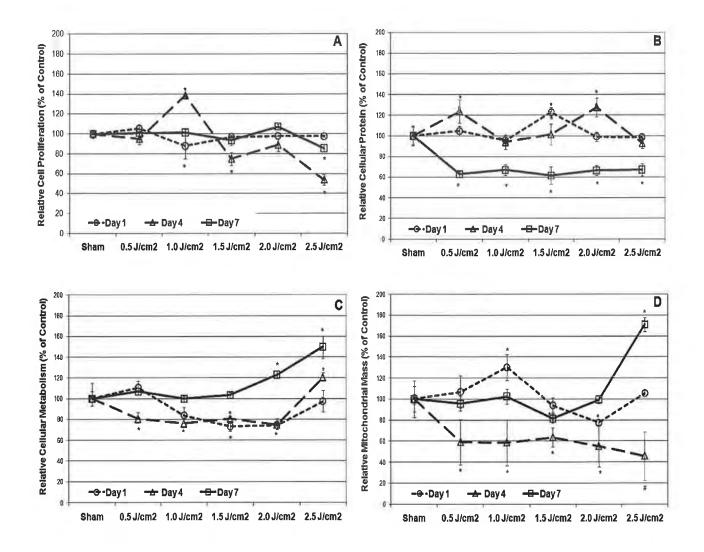


Figure 3.17: Simulated solar radiation-induced changes in cell proliferation, protein content, cellular metabolism and mitochondrial mass in spontaneously immortalized human keratinocytes (HaCaT cells) 1 day (circle), 4 days (triangle) and 7 days (square) post multiple sub-lethal irradiations. (A) Relative Cell proliferation was assessed by the Alamar Blue assay. (B) Relative Cellular Protein content was measured using the Biorad assay. (C) Relative Cellular metabolism was investigated using Biolog Redox Dye B. (D) Relative Mitochondrial mass was measured using the MitoTracker Green fluorescence dye uptake. Results are expressed as a percentage of sham-irradiated control cultures. * indicates significant difference compared to control (p < 0.05). The data represents the mean +/- SD of three independent experiments.

3.2. DNA analysis

3.2.1. Analysis of mtDNA deletion

Total DNA from HaCaT cells was used to analyse mtDNA⁴⁹⁷⁷ and the solar radiation exposure associated mtDNA deletion, mtDNA³⁸⁹⁵ (For details, please refer to Table 2.3). DNA concentration was adjusted prior to PCR analysis in all the samples. The 18S nDNA region amplification was performed as an index of template DNA loading in PCR reactions. The amplification of a conserved mtDNA region (D-loop) using primers detailed in Table 2.3 was performed to determine relative mitochondrial genome frequency (Figure 3.18).

Exposure to 1.0 J/cm² SSR yielded the first detectable increase in mtDNA⁴⁹⁷⁷ and mtDNA³⁸⁹⁵. Maximal induction of both mtDNA deletions were observed in 2.0 J/cm² and 1.0 J/cm² irradiated cells for the mtDNA⁴⁹⁷⁷ and mtDNA³⁸⁹⁵, respectively (Figure 3.18). Sham-irradiated control cells also presented with a basal level of both deletions (Figure 3.18). A dose dependent increase in the common deletion was observed in cell exposed to up to 2.0 J/cm² irradiations. The highest doses included in this study (2.5 J/cm²) led to a decrease in the relative amount of Δ mtDNA⁴⁹⁷⁷, 24 hours post irradiation (Figure 3.18).

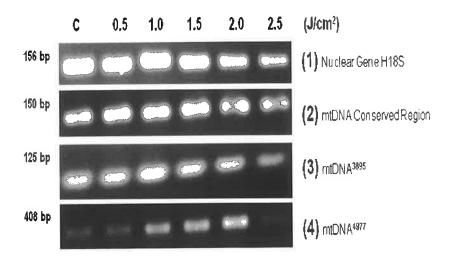


Figure 3.18: Multiple sub-lethal simulated solar radiation irradiations generate mtDNA deletions in HaCaT cells 24 hours post irradiations. Cells were irradiated with total doses of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 J/cm² of SSR delivered in fractions of 0.5 J/cm² irradiations every 12 hours. (1) PCR amplification of the 18S nuclear gene and (2) mtDNA D-loop (conserved region) amplification were performed as controls for DNA initial template homogeneous loading. (3) mtDNA³⁸⁹⁵ (solar radiation irradiation-associated deletion) and (4) mtDNA⁴⁹⁷⁷ (Common deletion) PCR analysis. Agarose gel image representative of three independent experiments.

3.2.3. Mitochondrial genome frequencyanalysis by qRT-PCR

To evaluate whether multiple SSR caused alterations in the mtDNA content in HaCaT cells, the relative mtDNA/nDNA was analysed 1 day post SSR exposures by RT-PCR. Two mtDNA regions including the Cytochrome *b* (Cyt b) and the NADH dehydrogenase 5 (ND5) genes were quantified using SYB green and normalized against the single copy cystic fibrosis nuclear gene (CF) amplification, as previously described (Wong and Cortopassi, 2008).

As shown in Figure 3.19B, both major and minor arc mtDNA region amplification presented similar trends 24 hours post irradiation except post 1.0J/cm². Approximately 20% increase in mtDNA copy number was observed in 1.0, 2.0 and 2.5 J/cm² cumulatively irradiated HaCaT cells using the ND5/CF ratio analysis (Figure 3.19B). Similarly, a maximum of 20% decrease in mtDNA/nDNA ratio was observed when the mitochondrial conserved region amplification (Cyt b) was quantified by RT-PCR and normalized against CF in 1.5 J/cm² irradiated HaCaT cells (Figure 3.19B). Maximum increase or decrease in mtDNA/nDNA were approximately 0.3 fold of that observed in sham irradiated cell, in our *in vitro* cellular system, using either ND5/CF or Cyt b/CF amplifications (Figure 3.19B).

Direct correlation was observed between the metabolic activity and the relative ND5 region frequency in cells irradiated with up to $1.5 \text{ J/cm}^2 \text{ SSR}$. Higher doses (2.0 and 2.5 J/cm^2) were found to induce an inverted correlation between mtDNA content and redox state 24 hours post irradiations in HaCaT cells (Figure 3.19C). The increase in mitochondrial mass, 24 hours post all irradiation regimes, directly correlated with the relative mtDNA content quantified by qRT-PCR using Cytochrome *b* region amplification (Cyt b) normalized by CF gene amplification (Figure 3.19D).

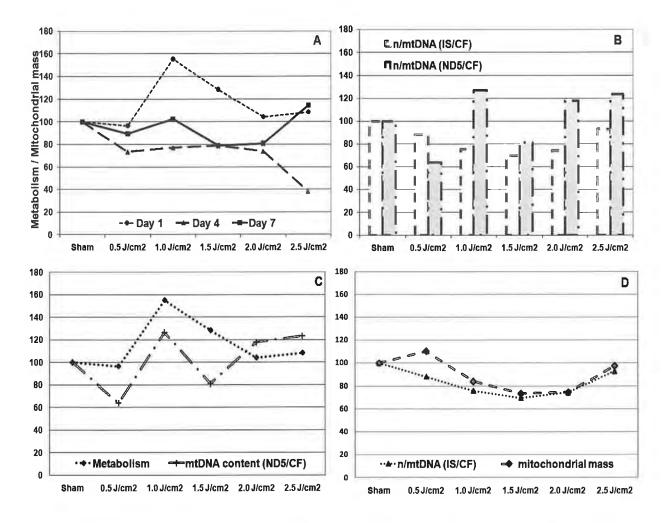


Figure 3.19: Mitochondrial DNA content and metabolic activity normalized by relative mitochondrial mass in HaCaT cells subjected to multiple sub-lethal SSR. (A) Metabolic activity (Biolog Redox assay normalized by the total mitochondrial mass measured by MitoTracker Green assay). (B) mtDNA frequency assessed by real-time quantitative PCR comparing the amplification profiles of two mtDNA regions (IS and ND5) and a single copy nuclear gene amplification (Cystic Fibrosis). (C) Correlation between mtDNA frequency measured by ND5/CF ratio and the metabolic activity measured by Biolog Redox Dye Assay. (D) Correlation between mtDNA/nDNA quantification by Cyt b/CF amplification and the total mitochondrial mass measured by MitoTracker assay. Cyt b - Mitochondrial Cytochrome *b* gene quantification, ND5 – mitochondrial NADH-dehydrogenase gene quantification, CF – nuclear single copy gene for cystic fibrosis quantification. Measurements were taken 24 hours post irradiation. Results are expressed as a percentage of control and are representative of triplicate experiments.. * denotes significant differences as compared to sham irradiated cells (p<0.05).

3.2.4. Mitochondrial Gene Copy Number Variation

Two mtDNA regions present in the frequently deleted region (major arc) and in the region less frequently involved in large deletion events (minor arc) were quantified by qRT-PCR and normalized against the mitochondrial Cytochrome *b* (Cyt b) gene (located near the mitochondrial heavy strand origin of replication (O_H) and less frequently involved in mtDNA rearrangements). Results are presented as relative frequency of ND1 or ND5 normalized against Cyt b (Figure 3.20).

ND1 and ND5 relative quantification was comparable 1 hour post lower SSR doses (Figure 3.20A). Higher SSR doses (5.0 and 7.5 J/cm²) induced a deviation in ND1 and ND5 gene frequency (Figure 3.20A). Although relatively small, ND1 and ND5 differences in amplification were observed also 30 minutes post single 5.0 J/cm² irradiation (Figure 3.20B). SSR induced a more pronounced decrease in ND5 gene copy number at 24 hours post single 5.0 J/cm² irradiation dose (Figure 3.20B).

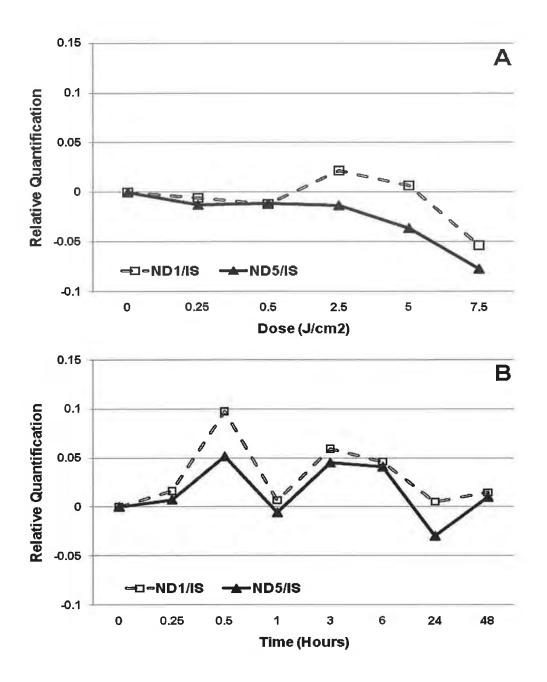


Figure 3.20: Amplification of mtDNA ND1 and ND5 gene regions in HaCaT cells post SSR. Two mtDNA regions located in the major arc (ND5) and minor arc (ND1) were quantified using SYBR green. (A) Cells were irradiated with increasing SSR dose and (B) irradiated with 5 J/cm² and assessed at different times points post irradiation Data are expressed as mean +- SEM (n=9).

SSR-induced increase in the relative frequency of mtDNA was observed mainly at 3 hours post irradiation, as measured with qRT-PCR. Furthermore, there was a tendency towards a decrease of mtDNA/nDNA ratio in the 0.25, 0.5 and 7.5 J/cm² irradiated HaCaT cells, 1 hour post irradiation (Figure 3.21C). The mtDNA content in the dose-curve studies did not correlate linearly with increasing SSR doses (Figures 3.21A and 3.21C).

As shown in Figure 3.21D, SSR irradiated cells displayed higher mtDNA/nDNA ratios representative of increased mtDNA levels induced by SSR exposure. mtDNA content peaked 0.5 hours post a single 5.0 J/cm² irradiation (Figure 3.21D).

The mtDNA amplification measurements using Picogreen assay did not directly correlate with the mtDNA/nDNA ratio quantified by qRT-PCR as the former analysis showed that 48 hours post 5.0 J/cm² irradiation the mtDNA content was only 0.5 fold of that measured in the control cells whereas the latter and more precise qRT-PCR quantification results demonstrated that an approximately 1.1-fold increase in the relative mtDNA/nDNA quantification was present 48 hours post SSR (Figures 3.21B and 3.21D).

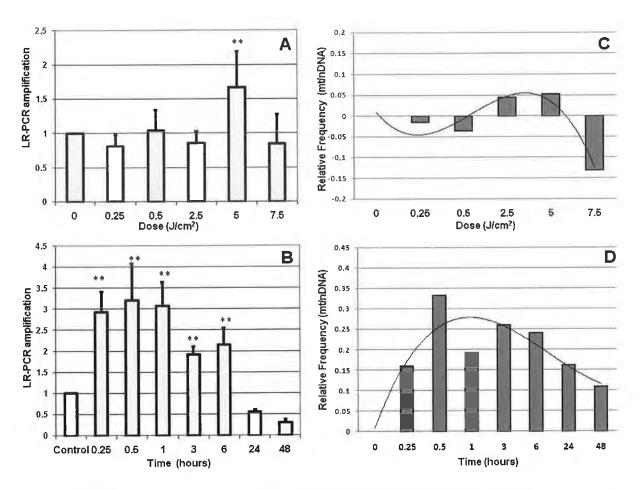


Figure 3.21: Total mitochondrial genome amplification and nDNA/mtDNA oscilations in HaCaT cells SSR. (A, B) mt genome 16 Kb long-range PCR product amplification measured by the Picogreen assay and (C,D) relative quantification of the mitochondrial genome (Cyt b region) normalized by the nuclear genome (single copy gene of Cistic Fibrosis) measured using the SYBR green assay (qRT-PCR). (A,C) Dose-curve (measured 1 hours post irradiation) and (B,D) time-course (post a single 5 J/cm² irradiation dose) of SSR-induced mitochondrial mass and mtDNA/nDNA changes in HaCaT cells. LR-PCR amplifications were calculated by normalizing the DNA amplification of sham-irradiated control cells using the Picogreen assay as described in Material and Methods. qRT-PCR of the mitochondrial genome region (Cyt b) was normalized by the amplification of the nuclear genome amplification (cystic fibrosis) (relative quantification) and expressed as a proportion of the sham-irradiated control cells mtDNA/nDNA ratio. The data were expressed as mean +- SEM (n=9). * denotes p<0.05 significantly different from sham-irradiated controls using Student's t-test.

3.2.5. Oxidative mitochondrial DNA Damage and Repair Assay

HaCaT cells were exposed to increasing SSR doses (0 to 7.5 J/cm²) and subsequently harvested one hour after irradiation for DNA damage analysis. The variation in mtDNA and nDNA damage and repair were measured using a QPCR assay. As shown in Figure 3.22, SSR-induced lesions were detected both in nDNA and mtDNA.

Nuclear DNA (DNA polymerase β gene) inhibition of the LR-PCR amplification, indicative of increased nDNA damage, was observed in dose-curve studies (Figure 3.22B). The highest nDNA lesion frequency was observed 1 hour post 2.5 J/cm² irradiation (Figure 3.22B). In contrast, the frequency of mtDNA lesion when compared to nDNA, was observed to not be significantly changed 1 hour post all SSR doses included in the present study (Figure 3.22B).

To assess the repair of SSR-induced DNA lesions, sub-confluent HaCaT cells were exposed to 5.0 J/cm² and DNA samples were analysed for lesion frequency at different time-points after irradiation. The 5.0 J/cm² dose was used in subsequent time-lapse studies, as it was found to induce the highest mtDNA 16 Kb LR-PCR product frequency, quantified by the Picogreen assay (Figure 3.22A). . HaCaT cells post SSR showed greater mtDNA lesion accumulation 3 hours post irradiation (Figure 3.22D). MtDNA repair was first apparent 6 hours post irradiation (Figure 3.22D). Time-lapse studies results also showed that a single 5.0 J/cm² irradiation induced nDNA damage which was observed to be peak at 48 hours post irradiation (Figure 3.22D).

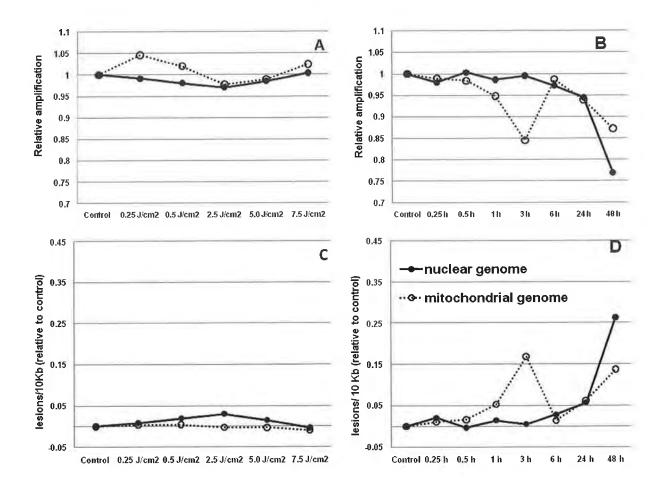


Figure 3.22: DNA damage and repair kinetics in HaCaT cells post SSR. (A, C) relative LR-PCR amplification of a 16.0 Kb mtDNA amplicon measured with the Picogreen assay and (B, D) amount of lesion per 1.0 Kb detected in the mitochondrial and nuclear genomes (with normalization of mitochondrial copy number). Calculated lesions per 1.0 Kb based on the inverted relationship between LR-PCR amplification efficiency and DNA lesions able to inhibit the enzyme activity, *in vitro*. (A, C) Dose-curve of SSR-induced mitochondrial and nuclear DNA damage. (B, D) Time-course of SSR-induced mitochondrial genomes. LR-PCR amplification observed for both nuclear and mitochondrial genomes. LR-PCR amplifications of nuclear (13.5 Kb of DNA polymerase β) and mitochondrial (8.9 Kb mitochondrial genome region) DNAs were amplified in triplicates, from 3 sets of independent experiments and measured using the Picogreen assay. Control cultures were SSR sham-irradiated. The data were expressed as mean +- SEM (n=9).

3.2.5.1. Mitochondrial DNA lesions and mitochondrial mass correlation

To investigate if mtDNA damage is accompanied by change in mitochondrial mass post SSR, the MitoTracker assay was performed following the same dose-curve and timelapse experimental designs used to assess the mtDNA lesions accumulation by QPCR. Lower SSR doses (up to 2.5 J/cm²) induced mitochondrial mass increase of approximately 80% of the mitochondrial mass measured in the sham-irradiated control cells (Figure 3.23A).

Interestingly, mitochondrial mass and mtDNA lesions accumulation oscillated following similar patterns up to 1 hours post single 5.0 J/cm² irradiation dose (Figure 3.23B). However, 3 hours post irradiation, an increase in mtDNA lesions ratio was observed in parallel to a 40% decrease in mitochondrial mass (Figure 3.23B). MtDNA lesions and mitochondrial mass followed inverted patterns (higher mtDNA lesions correlating with decrease in mitochondrial mass) up to 48 hours post a single 5.0 J/cm² irradiation (Figure 3.23B).

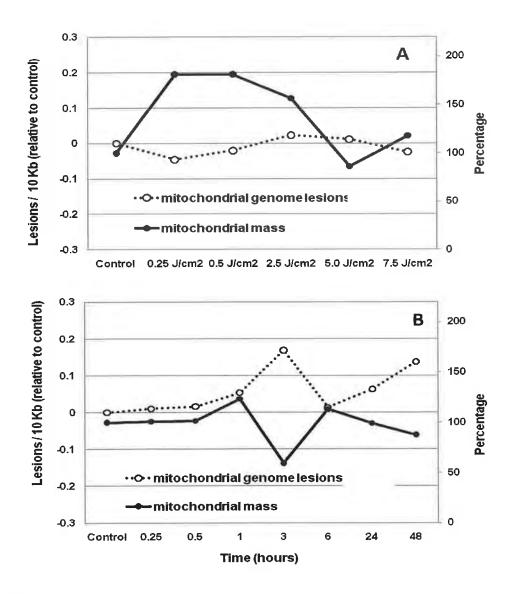


Figure 3.23: (A) Cells were irradiated with increasing SSR doses or (B) irradiated with 5.0 J/cm² and assessed at different times points post irradiation (from 0.25 up to 48 hrs post irradiation). Mitochondrial mass and mtDNA damage were assessed by Mitotracker assay and by QPCR, respectively. Mitochondrial mass results are expressed as a percentage of the sham-irradiated controls. Lesions of irradiated cells were calculated by normalizing the DNA amplification to sham-irradiated control cells. 8.9 Kb of the mitochondrial genome was amplified and the LR-PCR product was quantified using the Picogreen assay. Calculated mtDNA lesion per 1.0 Kb was based on the inverted relationship between the Taq polymerase efficiency and the mtDNA lesions able to inhibit the enzyme activity, *in vitro*.

3.2.6. Quantification of mtDNA³⁸⁹⁵

To confirm mtDNA damage accumulation, induced by SSR, the UVR-related mtDNA³⁸⁹⁵ was quantified using a Taqman assay as previously described (Harbottle and Birchmachin, 2006). MtDNA³⁸⁹⁵ was quantified using probes specific for the deletion junction region and normalized against (Cyt b) mtDNA cytochrome *b* region quantification.

Lower SSR doses (0.25 up to 2.5 J/cm²) induced a decrease in the mtDNA³⁸⁹⁵ whereas higher SSR doses (5.0 and 7.5 J/cm²) induced only a moderate increase in the deleted mtDNA relative to control (Figure 3.24A). Time course studies revealed that the mtDNA³⁸⁹⁵ frequency was decreased down to control levels 24 hours post a 5.0 J/cm² irradiation and that the deletion ratio was subsequently elevated, at 48 hours post irradiation (Figure 3.24B).

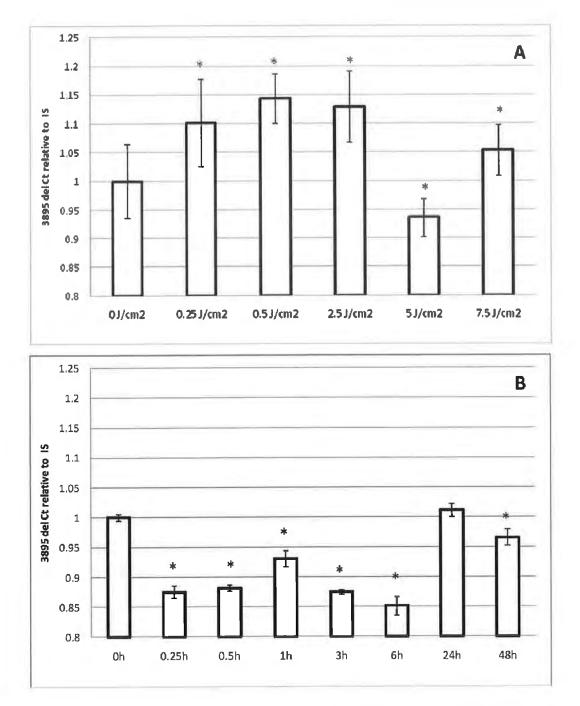


Figure 3.24: mtDNA³⁸⁹⁵ frequency in HaCaT cells post SSR. (A) dose-curve study of SSR and (B) time-course post 5.0 J/cm² SSR. mtDNA³⁸⁹⁵ was assessed by real-time PCR using a Taqman assay. Results are expressed as arbitrary units relative to the sham-irradiated control cells basal level of mtDNA³⁸⁹⁵ (set as 1). The mtDNA³⁸⁹⁵ deletion content was normalized against the mitochondrial Cytochrome *b* levels measured by Taqman assay in the same samples, to correct for the mtDNA content variation.

3.3. Mitophagy Analysis

3.3.1. Lysosome Quantification

All SSR doses induced significant total cell area reduction in A375 cells 4 hours post irradiation (Figure 3.25A) when cultured in complete growth medium (4.5 mg/l glucose, 2 mM glutamine, 1 U/ml penicillin/streptomycin). Confocal microscopy analysis of single cells allowed for the manual identification of cell and lysosome borders in superimposed confocal microscopy images from 7 Z-stacks. Interestingly, small (likely nascent) lysosomes (< 1 μ m) presented the highest increase in comparison with medium sized (from 1.0 – 2.0 μ m) and larger lysosomes (> 2 μ m) (likely autophagolysosomes) (Figure 3.25B). The number of small lysosomes in cells exposed to 2.5 J/cm² were 6-8 fold that found in sham-exposed cells (Figure 3.25B).

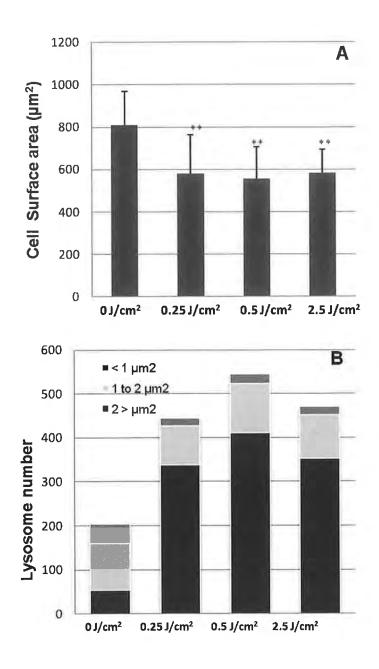


Figure 3.25: A375 cells were exposed to simulated solar radiation doses of either 0, 0.25, 0.5 or 2.5 J/cm². 4 hours post exposure / sham exposure, cell populations were assessed for (A) cell area, analysed by confocal microscopy and (B) lysosome number and size (per 50 cells analysed). Lysosome size was determined by measuring individual lysosome size plotted against the total number of lysosomes measured, determined by confocal microscopy. Experiments were performed in triplicate and at least 50 cells were analysed per simulated solar radiation irradiation dose. (*) p < 0.05; (**) p < 0.01.

A dose-dependent increase in lysosomal staining was observed in A375 cells 4 hours post SSR exposure (Figure 3.26). Sham irradiated cells presented a smaller number of lysosomes that were both smaller and more discretely stained in comparison with SSR exposed cells. A375 cells exposed to longer periods of SSR displayed increased lysosomal staining that typically was more diffusely stained, with some LTG-stained cell areas having no discernable boundary at all (Figure 3.26). Furthermore, the mitochondria in A375 cells exposed to longer periods of SSR display fusion, as observed by the morphologically complex organization of the mitochondrial network into hyperfused, branched mitochondria (Figure 3.26). Cells exposed to the highest dose (2.5 J/cm²) of SSR appeared to present with a greater number of small lysosomes in proximity to the mitochondrial network (Figure 3.26). Similarly, in A375 cells irradiated with 2.5 J/cm², mitochondria appeared swelled with diffuse staining and less discernable boundaries (Figure 3.26).

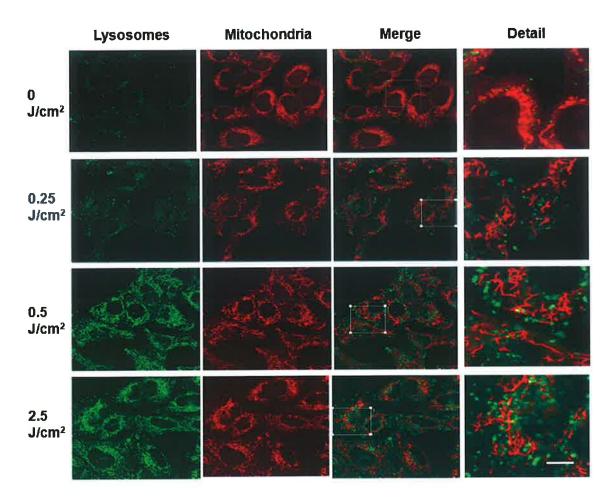


Figure 3.26: Fluorescence confocal microscopy analysis of A375 cells 4 hours post exposure to simulated solar radiation doses of either 0, 0.25, 0.5 or 2.5 J/cm². Mitochondria and lysosomes were stained with MitoTracker Red and LysoTracker Green respectively. In detail, the simulated solar radiation-induced morphological changes in mitochondria and the number of acidic organelles (lysosomes/ autophagossomes). Images are representative of triplicate experiment. Scale bar: $10 \mu m$.

3.3.2. Mitochondrial Morphology

The mitochondrial network in sham irradiated A375 cells showed a range of morphology including: small fragmented mitochondria; longer filamentous-like structures; rounded structure and bigger organelles (Figure 3.27A). The mitochondrial network appeared highly inter-connected in A375 cells post exposure to SSR, presenting filamentous hyper-fused mitochondria and a sub-population of rounded and smaller isolated mitochondria (Figure 3.27B).

Cells sham exposed to SSR and incubated with 0.5 µM FCCP (mitochondrial OxPhos uncoupler) for 2 hours exhibited fragmented and faintly stained mitochondria in comparison with mitochondria from control cells (Figure 3.27C). Mitochondrial staining accumulates in the inter-membrane mitochondrial space in a mitochondrial membrane dependent way, and as FCCP is capable of depolarizing plasma and mitochondrial membranes, fainter staining and increased fiSSRon of the mitochondrial network was expected. As well as an increase in mitochondrial fission, A375 cells supplemented with FCCP showed diffuse cytoplasmic MTR staining and mitochondrial swelling with an increase in MTR accumulation post SSR (Figure 3.27D).

Creatine supplementation and glutamine starvation were found to induce mitochondrial fragmentation, inter-mitochondrial membrane disorganization and diffuse MTR cytoplasmic staining in A375 cells post SSR (Figures 3.27F and 3.27G).

Neither glutamine starvation nor supplementation induced significant morphological changes in sham irradiated cells (Figures 3.27G and 3.27I). However, following SSR exposure, glutamine supplemented cells presented increased mitochondrial fusion resulting in an organized and complex filamentous mitochondrial network with apparent heterogeneous MTR staining along the hyper-fused mitochondria (Figure 3.27J), whereas glutamine starvation resulted in swollen and fragmented mitochondria and diffuse cytoplasmic MTR staining (Figure 3.27H).

Melanin supplementation of sham exposed cells induced two heterogeneous mitochondrial subpopulations: (i) faintly stained smaller, elongated mitochondria and (ii) bigger, more brightly stained mitochondria (Figure 3.27K). Following SSR exposure, a more homogeneous and brightly stained hyper-fused mitochondrial network was observed in the melanin supplemented A375 cells (Figure 3.27L).

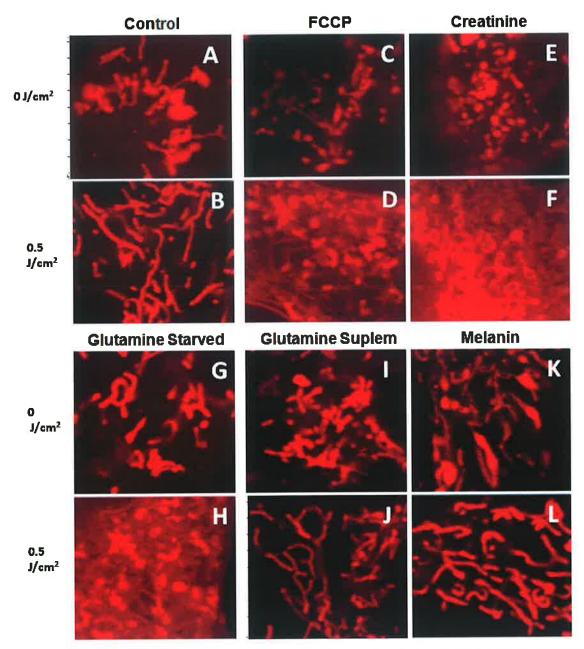


Figure 3.27: Fluorescence confocal microscopy analysis of mitochondrial morphology dynamics using MitoTracker Red stainning in A375 cells 4 hours post either sham SSR exposure (A,C,E,G, I, K) or post 0.5J/cm² SSR exposure (B, D, F, H, J and L) . Cell populations were either supplemented with 0.5 mM FCCP (C,D), 100mM creatine (E,F), 0.6 mM glutamine (G,H), 6 mM glutamine (I,J), 10 mg/ml melanin (K,L) or maintained as control (A,B - normal growth medium). Images are representative of triplicate experiments with a minimum of 100 cells analyzed.

In order to confirm that the cellular response to solar radiation is dependent on mitochondrial activity and morphological dynamics, A375 cells were supplemented with either FCCP, creatine, melanin or glutamine (the latter included starvation) and their effects on mitochondrial network dynamics and mitophagic induction determined. Creatine treated cells (without SSR) showed a significant increase in cell diameter, a significant decrease in mitochondrial and lysosomal fluorescence and significantly increased lysosomal to mitochondrial fluorescence ratio in comparison with all other cell treatments (Figures 3.28A, 3.28B, 3.28C and 3.28D).

A375 cells that were SSR exposed and either FCCP treated or glutamine starved presented a pronounced increase in cell diameter, a significant decrease in mitochondrial and lysosomal total fluorescence per cell and a modest, though significant, increase in the lysosomal to mitochondrial fluorescence ratio, suggesting a comparable reduction in the total number of both organelles (Figure 3.28).

Interestingly, melanin treated cells exposed to SSR presented an increase in cell diameter, a modest reduction in mitochondrial and lysosomal fluorescence, but no statically significant change in the lysosome to mitochondria ratio, compared to similarly melanin treated and sham SSR exposed cells (Figures 3.28A, 3.28B, 3.28C and 3.28D).

Furthermore, cells supplemented with glutamine did not present a change in cell diameter, mitochondrial or lysosomal fluorescence, nor lysosome to mitochondrial ratio post SSR exposure. Similar results were observed in the UV-protective melanin supplemented cells (Figures 3.28A, 3.28B, 3.28C and 3.28D).

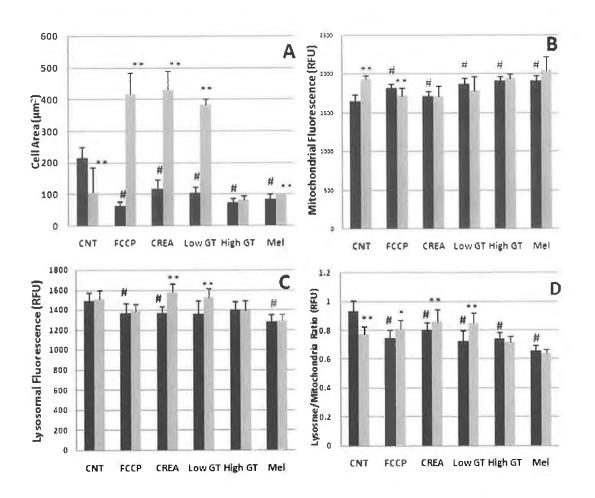


Figure 3.28: Confocal microscopy analysis of cell swelling and organellar fluorescence post SSR sham and $0.5J/m^2$ exposure in A375 cells pretreated with a range of mitochondrial activity modulators. Cells were assessed for hours post exposure/ sham exposure for (A) cell area; (B) mitochondrial mass; (C) lysosome mass and (D) approximate lysosome to mitochondria fluorescence ratio. Contrast faze images were used to isolate regions of interest from individual cells and MTR and LTG were used as organelles specific markers of mitochondria and lysosomes, respectively. Cell populations with either supplemented with 0.5 mM FCCP, 100mM creatine, 0.6 mM glutamine, 6 mM glutamine, 10 mg/ml melanin or maintained as control (normal growth medium). Relative fluorescence per cell area was acquired using the LMS 510 analysis software. Results are presented as mean ± SD from triplicate experiments where 10 images were acquired per treatment and at least 50 cells analyzed per treatment. (#) p < 0.05 vs. sham control; (**) p < 0.05 vs. SSR irradiated control.

3.3.3. Co-localization Analysis

Fluorescence confocal microscopy of MTR and LTG co-stained A375 cells post SSR exposure indicates that mitochondrial hyper-fusion occurred in melanin and glutamine supplemented cells (Figure 3.29A) in contrast to mitochondrial network fragmentation that was observed in both FCCP and creatine pre-treated cells and in glutamine starved cells (Figure 3.29A).

Lysosomes and acidic organelles were observed in the immediate vicinity of the hyperfused mitochondrial network though organelle co-localization was not discernable (Figure 3.29B).

A375 cells supplemented with creatine presented fragmented mitochondria post SSR exposure (Figures 3.26 and 3.27). The fragmented mitochondria co-localized with acidic organelles (lysosomes and autophagosomes) as indicated by a yellow fluorescence (quantified in Figure 3.29D) indicative of increased mitophagy. Numerous larger and more brightly stained lysosomes were also observed (Figure 3.29C), potentially involved in active autophagic and mitophagic processes.

Quantitative co-localization analysis was performed using the confocal microscopy software, Image Processing Kit 4.0.25 software (Carl Zeiss), which employs specialized algorithms to estimate the degree of overlap of fluorescence signals (Pearson's correlation). The Pearson's correlation coefficient is a pattern recognition coefficient used to estimate co-localization. The Pearson's values range from 1.0 to - 1.0; with a 107

value of 1.0 meaning significant correlation while values of 0 or less indicate complete negative correlation (Manders *et al.*, 1992). From Figure 3.28D it can be observed that significant correlation between mitochondrial and lysosomal fluorescence are present in FCCP, creatine and low glutamine treated A375 cells, 4 hours post 0.5 J/cm² SSR exposure.

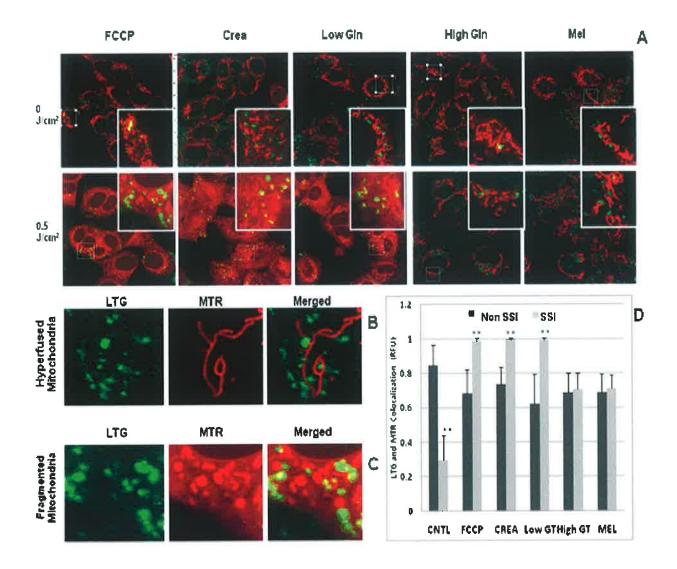


Figure 3.29: Confocal Microscopy analysis of MitoTracker Red and LysoTracker Green fluorescence colocalization, indicative of mitophagy. A375 cells were analysed 4 hours post SSR exposure to 0.5J/cm² or post sham irradiation. (A) Cells were supplemented with either 0.5 mM FCCP (FCCP), 100 mM creatine (CREA), 0.6 mM glutamine (low GT), 6mM glutamine (High GT) or 10 mg/l melanin (MEL). (B) Detailed fluorescence images of 6 mM glutamine-supplemented cells illustrating hyper-fused mitochondria escaping mitophagy as no mitochondria-lysosome co-localisation (yellow fluorescence) is observable in the merged image. (C) Detailed fluorescence images of FCCP-supplemented cells illustrating fragmented mitochondria actively undergoing mitophagy as observed by mitochondria and lysosome co-localization in the merged image (yellow). (D) Lysosomes and mitochondrial co-localization (Relative Fluorescence Units – RFU) semi-quantitatively measured from 7 overlapping z-stack confocal images acquired from 50 or more cells in triplicate experiments. **, p<0.01 vs.sham irradiated cells.

Chapter 4: Discussion

4.1. Cellular Response

4.1.1 Cell lines

In the present study, the response of spontaneously immortalized human keratinocytes (HaCaT), human malignant melanoma (A375) and human amelanotic melanoma (C32) cells to SSR was evaluated. The study employed apoptosis, cell viability, cellular metabolism, mitochondrial mass and mitochondrial membrane potential analysis.

A cascade of cellular and molecular adaptations is initiated in response to UVRinduced damage, namely ROS formation (Cooper *et al.*, 2009), lipid and protein peroxidation (Negre-Salvayre *et al.*, 1992), DNA damage (Gruenert and Cleaver, 1981), DNA synthesis arrest and single-strand breaks (Fornace *et al.*, 1976; Carty *et al.*, 1994), as well as cell cycle arrest (Pavey *et al.*, 2001). These alterations are expected to alter cellular parameters such as antioxidant content and DNA repair.

Epidermal keratinocytes are the cell type most adapted to solar radiation and are more resilient to DNA and molecular damage than other cell types, such as melanocytes (Cooke *et al.*, 2000; Penland, 2008). The HaCaT keratinocytes employed in the present study grow as a monolayer and were established from

normal epidermis (Boukamp *et al.*, 1988). Although possessing chromosomal abnormalities and UV-induced mutations on the p53 gene this cell line is considered a good *in vitro* model for squamous cell carcinoma (SCC) studies (Boukamp *et al.*, 1988).

The melanoma derived-cell lines are the most commonly used cell models for antitumoural drug screenings (Tveit *et al.*, 1981; Grottke *et al.*, 2000). It has also been reported that these cell lines vary widely in their sensitivity to UVR stress (Chenevix-Trenh *et al.*, 1992; Kowalczuk *et al.*, 2006). Additionally, it has been observed that some skin-cancer derived cell cultures present increased cell proliferation, protein expression and antioxidant production in response to UVR (Campbell *et al.*, 1993; Mattei *et al.*, 1994; Hedley *et al.*, 1998; Sander *et al.*, 2003).

In the present study two human amelanotic melanoma cell lines were also employed, namely C32 and A375 cell lines. Melanoma arises from transformation of epidermal melanocytes in skin, which is a relatively hypoxic tissue (Govindarajan *et al.*, 2003; Hoek *et al.*, 2004). The association between melanoma and solar UVR is causative for at least a subset of tumours and heritable mutations (CDKN2A, CDK4) and polymorphisms have been reported in this skin cancer type (Monzon *et al.*, 1998; Borg *et al.*, 2000; Kumar *et al.*, 2001).

4.1.2. Detection of Cell Death

The elimination of UV damaged cells by apoptosis or necrosis is important in eliminating potentially tumourigenic cells in the epidermis. Apoptosis is associated 111

with p53 expression which induces the expression of Bax family proteins (Adams and Cory, 1998). These proteins lead to mitochondrial outer membrane permeabilization and cytocrome *c* release from the inter-membrane space into the cytoplasm, culminating in caspase activation and programmend cell death (Chipuk *et al.*, 2004).

Apoptosis is observed relatively early post UVB irradiation in HaCaT cells (Henseleit *et al.*, 1997). It has also been reported that an increase in ROS production results from UVR irradiation in HaCaT cells (50 mJ/cm² UVB) leading to maximal p53 expression and apoptosis levels 24 hrs post irradiation (Paz *et al.*, 2008). The p53 mutation is found at dipyridine sites typical of UV-induced damage in HaCaT cells and the mutation is possibly also associated with the immortalization of this cell line (Lehman *et al.*, 1993).

In the present study conditions, a 2.5 J/cm² dose of SSR was observed to decrease apoptosis levels in melanoma derived cell lines in vitro (A375 and C32, Figures 3.1 and 3.2). Representative apoptosis and necrosis percentages are depicted in Figures 3.1, 3.2 and 3.3, demonstrating a maximal increase in necrosis in HaCaT cells irradiated with 2.5 J/cm² and in C32 cells SSR-sham irradiated (basal necrosis level). SSR affects cell types differently, with more pronounced decrease in both apoptotic and necrotic cell levels having been observed in the 2.5 J/cm² irradiated A375 cells.

4.1.2. DNA quantification

DNA content is the most significant parameter for the accurate assessment of cell numbers (West *et al.*, 1985; Gillies *et al.*, 1986) whereas the Picogreen assay is the most sensitive DNA binding dye in comparison to Hoechst and DAPI (Singer *et al.*, 1997; Rengarajan *et al.*, 2002). The Picogreen assay has been previously used to accurately estimate the cell number present in tissue engineering scaffolds (Ng *et al.*, 2005). Moreover, reduction in DNA content has been associated with apoptosis induction or reduction in the mitotic index (Grove *et al.*, 1969; Nicoletti *et al.*, 1991; Darzynkiewicz *et al.*, 1992).

Reduction in DNA content measured by Picogreen staining may be due to total cellular DNA reduction, reduction in double-stranded DNA (as induced by increase in gene expression) or progressive induction of nucleic acids damage (Fomace *et al.*, 1989; Galitski *et al.*, 1999). Indeed, increase in energy levels of UV radiation has been described in association with reduced fluorescence staining of both DNA and RNA in HaCaT cells (Mayne and Lehmann, 1982). Even low UV dose (between 0 and 0.09 J/cm²) have been reported to induce the formation of DNA strand breaks measured by the comet assay (Lehmann *et al.*, 1998).

Furthermore, the processes of DNA expression and replication, both of which influence the total DNA content measured by the Picogreen assay, involve epigenetic processes such as DNA methylation and histone modifications, which are also known to contribute to the phenotype of transformed cells (Esteller, 2007).

In the present study it has been observed that SSR induced significant increase in total DNA content in A375 and HaCaT cells whereas significant decrease in the same parameter was observed in C32 cells (Figure 3.5). Interestingly, A375 presented a decrease in the proportion of cells undergoing apoptosis and necrosis and would explain the observed increase in DNA content (possibly indicative of cell cycle arrest as no significant increase in cell proliferation was observed, as discussed below).

Additionally, the Picogreen assay has been used to estimate cell number more efficiently than the widely used Alamar Blue and MTT assays as the metabolic activity measured by such dyes do not always accurately correlate with total cell number (Goegan *et al.*, 1995; Petty *et al.*, 1995).

For the aforementioned reasons, in the present study, the metabolic activity assays (Alamar Blue, Biolog and MTT) as well as the mitochondrial mass and mitochondrial membrane measurements were normalized against the total DNA content before being expressed as a percentage of the sham-irradiated control cells.

4.1.3. Protein Quantification

Protein content variation post SSR can be due, amongst other reasons, to altered protein expression or protein degradation rates. UVR is known to increase antioxidants, DNA repair and mitochondrial proteins associated with energy 114 production (Maytin, 1992; Neocleous *et al.*, 1997, Seite *et al.*, 2004; Kossodo *et al.*, 2004). UVR has also been reported to induce protein peroxidation, lysosome damage and decreased proteasome activity (oxidized protein degradation) (Kochevat, 1990; Brunk *et al.*, 1995; Boya *et al.*, 2003). SSR increased total protein content in C32 cells, 4 hrs post irradiation whereas no significant change in protein content were observed, in either A375 or HaCaT cells, at the same time point (Figure 3.6). This increase in protein content but not DNA content in C32 cells suggests an increase in gene expression and/or protein translation in these melanoma cells however such parameters were not evaluated in the present study.

4.1.4. Metabolic Activity

To further demonstrate that SSR induced changes in cellular metabolic activity, Alamar Blue, Biolog and MTT assays were performed. These dyes are used to measure the metabolic activity using tetrazolium-based reagents that are reduced to a soluble formazan product by live and respiring cells (Scudiero *et al.*, 1988).

The Alamar Blue assay uses a nontoxic fluorometric REDOX indicator that is reduced by FMNH2, FADH2, NAD(P)H and cytochromes, which determine the metabolic activity of cells (Ihalin *et al.*, 2003). It has been reported that UVR reduced activity of the mitochondrial NADH dehydrogenase in a fibroblast model studied by mitochondrial enzyme activity assays (Lofgren and Soderberg, 1998). NADH dehydrogenase has also been suggested to be the main reducing molecule detected by the assays that measure cellular metabolic activity such as MTT, Alamar Blue and Biolog Redox (Huet *et al.*, 1992) (Figure 3.10).

Decreases in cell division and cell cycle arrest were suggested to enable DNA repair or to more efficiently direct damaged cells to apoptosis (Di Leonardo *et al.*, 1994). It has also been reported that UVA irradiation reduces mitochondrial activity in keratinocytes, *in vitro*, even post irradiation with doses that were lower than the minimal erythemal dose (Leccia *et al.*, 2001).

Using the Alamar Blue assay to assess the metabolic activity it was observed that 0.5 J/cm² SSR decreased and increased metabolic activity in A375/HaCaT and C32 cells, respectively (Figure 3.7). Cellular metabolism measured by the MTT and Biolog Redox assays did not present comparable results. The Biolog assay only detected a significant reduction in cellular metabolism in A375 cells irradiated with 0.5 J/cm² and significant increase in the same parameter in C32 cells irradiated with 2.5 J/cm² SSR dose. Meanwhile, the MTT assay only detected a significant increase in metabolic activity in C32 cells irradiated with 0.5 and 2.5 J/cm².

These disparate results may reflect differences in the cellular metabolite that is specifically measured by each dye. Despite these differences, all dyes showed higher metabolic activity in C32 cells post SSR, suggesting that real metabolic changes are present in this cell line. In addition, the mitochondrial mass and mitochondrial membrane potential were also increased in C32 cells (discussed in detail below).

4.1.7. Mitochondrial Analysis

4.1.7.1. Measurement of Mitochondrial Mass

It has been reported that mitochondria represent a target site of UVR phototoxicity. Several studies have demonstrated that UVR impairs mitochondrial activity (Djavaheri-Mergny *et al.*, 2001).

ROS, a byproduct of cellular respiration, is known to induce an increase in mitochondrial mass as well as to induce the appearance of large scale mtDNA deletions in UVR irradiated cells (Lu *et al.*, 1999; Lee *et al.*, 2002).

Mitochondrial population size varies with different energy demands and from cell type to cell type. The number of mitochondria per cell is controlled by mitochondrial biogenesis which is regulated by multiple signalling and controlling factors which are still a matter of on-going research (Moraes, 2001; Wredenberg *et al.*, 2002; Nisoli *et al.*, 2003).

To add complexity to the analysis of mitochondrial populations post SSR, the mitochondrial population is usually heterogeneous in size, mitochondrial membrane polarization and mobility, especially post oxidative stress such as induced by UVR (Cossarizza *et al.*, 1996; Chen *et al.*, 2005).

Importantly, in the present study, the MitoTracker Green (MTG) dye was used to assess mitochondrial population variation post SSR. MTG accumulates intramitochondrially, independent of MMP. Indeed it was observed, in the present 117 study, that melanoma-derived cell lines behave differently, either increasing or decreasing mitochondrial mass, in response to SSR (C32 and A375 cells, respectively) (Figure 3.10).

The observed increase in mitochondrial mass in C32 cells post 2.5 J/cm² irradiation and the decrease in the same parameter in A375 cells was corroborated using a second mitochondria-specific dye (Mitotracker Red, that accumulates proportionally to MMP) measured by flow cytometry (Figure 3.13), suggesting that an increase in mitochondrial mass was followed by a concomitant increase in mitochondrial polarization in response to SSR in the cell lines analysed. Additionally, a time-dependent change in mitochondrial mass was observed with mitochondrial mass peaking (100% increase) 20 hours post 1.0 J/cm² SSR dose and 96 hrs post irradiation of 0.5 J/cm² in C32 cells (Figure 3.10 and 3.11).

Several *in vitro* studies showed that change in mitochondrial mass occurred in response to UVR stress. However, no direct dose-dependent pattern in mitochondrial mass variation could be drawn from the present observations. Changes in mitochondrial mass are dependent on multiple factors such as nutrient and energetic sources availability, coordinated expression of protein associated with mitochondria fusion and fission, cellular bioenergetics status, mitochondrial membrane potential and antioxidants production (Izquierdo *et al.*, 1995; Wu *et al.*, 1999). Further studies would be necessary to elucidate the mechanisms of mitochondrial mass increase in response to SSR.

4.1.7.2. Measurement of mitochondrial membrane potential

In parallel to mitochondrial mass variation, mitochondrial membrane depolarization by SSR was assessed using TMRM dye accumulation in the mitochondrial inter-membrane space.

The cationic TMRM enables the analysis of intact mitochondria and accumulates in the mitochondria, in a MMP-dependent manner, since the mitochondrial matrix is the most negatively charged cellular organelle (Scaduto and Grotyohann, 1999).

Mitochondrial hypo-polarization can be due to reduced respiratory chain activity, increase in MMP dissipation such as ATP-synthase activity or decoupling of respiratory activity from proton motive force generation (ie. proton leak across the membrane or reduced activity of proton pumping complexes) (DiDonato, 2000; Tedeschi, 2005; Katoh *et al.*, 2008).

Mitochondrial depolarisation can also promote apoptotic and necrotic cell death post oxidative stress (Suematsu *et al.*, 2003; Kujoth *et al.*, 2005). Mitochondrial depolarization was pronounced in A375 cells exposed to both 0.5 and 2.5 J/cm² of SSR whereas the apoptosis levels were not significantly increased in 0.5 J/cm² irradiated cells (6.2 % versus 4.0% in sham-irradiated control cells – Figures 3.14 and 3.15). This observation is important as it is known that cell survival or death post solar radiation may be controlled by mitochondrial retro-signaling associated with apoptosis initiation or in supplying increased cellular ATP demands (Löfgren and Söderberg, 2001; Jacobson *et al.*, 2001).

Moderate mitochondrial depolarisation was observed in A375 and HaCaT cells 24 hours post SSR (Figure 3.14). In C32 cells an increase in mitochondrial function and cellular proliferation was observed in response to low-dose SSR. The sustained hyper-polarisation of C32 mitochondria, 4 hours post SSR suggests that the mitochondrial population in this cell line has a higher tolerance to SSR than in the other cell lines analysed. Hence, mitochondrial mass correlates with MMP and cellular metabolism in all cell types analysed.

4.1.8. Cell Density Manipulation and SSR response

Given the importance of ROS production and mitochondrial function in regulating response to solar radiation damage in skin cells, it was hypothesised that the degree of oxidative stress may also be impacted upon by changes in cell microenvironment, modelled in the present study through changing *in vitro* cell culture density (Experimental Design 2 – Table 2.1). Cell population density was observed to modulate change in ROS and MMP post SSR (Figures 3.15 and 3.16). To confirm that observed SSR-induced changes were not associated with specific cell density response to stress and injury, two different cell densities were employed for every experimental analysis.

4.1.8.1. Mitochondrial mass analysis

Mitochondrial mass variation post SSR was notably independent of cell density. In the dose-effect studies it was found that 0.25-2.5 J/cm² irradiation significantly

increased mitochondrial mass, a reponse which further increased with time, as observed post 5.0 J/cm² irradiation (Figs 3.14A and 3.15A). These results were in accordance with previous studies in which sub-lethal levels of oxidative stress caused an increase in mitochondrial mass in human fibroblasts and lung cancer cells (Wright *et al.*, 1994; Lee *et al.*, 2002). In addition, abnormal proliferation of mitochondria and mtDNA generally occurs in aging human tissues and in patients with mitochondrial myopathy (Pieri *et al.*, 1993; Aure *et al.*, 2006).

Mitochondrial proliferation depends on coordinated expression of nuclear and mitochondrial genes mediated by ROS generated in the mitochondrial respiratory chain (Palmeira *et al.*, 2007). Complex I (NADH-Q-reductase) and complex III (ubiquinone reductase) have been identified as the main contributors of endogenous ROS in the cell (Kushnareva *et al.*, 2002; Chen *et al.*, 2003). In addition, nitric oxide has been found to mediate mitochondrial biogenesis in various cell types (Nisoli *et al.*, 2003).

4.1.8.2. ROS analysis

ROS plays an important role in cellular signalling and regulating gene expression, cell proliferation and, if excessively produced, will induce mitochondrial membrane depolarization and the latter has been associated with mitochondrial recycling (mitophagy) and apoptosis (Zhang *et al.*, 2001; Paz *et al.*, 2008; Nakai *et al.*, 2008; Kim and Choi, 2008).

ROS levels peaked 24 hours post irradiation in cells seeded at both densities but only returned to control levels in HD seeded cells by 48 hours post SSR ROS were significantly decreased in an inverted dose manner in HD cells (Figure 3.16B). Similar to the present study, Bakondi *et al.* (2003) showed that cell-densitydependent signals modulate peroxynitrite, hydrogen peroxide and superoxide resistance in HaCaT cells.

Elevated ROS levels were likely associated with the concomitantly observed change in MMP post SSR that may compromise the steady-state ROS detoxification in these cells. The present study results are in accordance with observations from Lee *et al.* (2002) who reported that ROS formation and MMP control were associated with reversible phosphorylation of cytochrome *c* oxidase. It has been suggested that increase in cellular Ca^{2+} activates a mitochondrial protein phosphatase which dephosphorylates cytochrome *c* oxidase, turns off the allosteric ATP-inhibition (turned on by high ATP/ADP ratios) resulting in mitochondrial membrane hyper-polarization and ROS formation (Lee *et al.*, 2002).

Interestingly, H₂O₂ treatment of HaCaT cells induced a decrease in mitochondrial mass and MMP, independently of cell density (Fig 3.15A and 3.15C). It has been previously reported that UVA-induced H₂O₂ synthesis is a metabolically active process that depends on mitochondrial complex III activity (Gniadecki *et al.*, 2000). Moreover, the same authors demonstrated that inhibition of mitochondrial proton flow by oligomycin decreased UVA-induced radical production and suggested that a mitochondrial independent ROS synthesis pathway exists in keratinocytes as

mitochondrial blockers did not completely inhibit cellular H_2O_2 production in response to UVA.

Additionally, the level of intracellular ROS was higher in sub-confluent cultures of retinal pigment epithelial cells, compared to confluent cultures that were treated with increasing concentrations of H_2O_2 (Jin *et al.*, 2001). Similar findings were observed in the increased expression of fibroblast growth factor-2 (FGF-2) (Wada *et al.*, 2001), highlighting the importance of ROS in cellular proliferation.

H₂O₂ and GSH treatment did not induce either ROS production or detoxification in LD seeded cells, further suggesting an increased ROS buffering capacity in HaCaT cells during log phase of growth. It has been reported that HaCaT cells present a very efficient adaptive response to enhanced oxidative stress represented by a 3fold increase in MnSOD following 50 mJ/cm² irradiation (Wiswedel *et al.*, 2007). However, at higher UV doses, MnSOD levels were reduced, leading to irreversible cell damage (Wiswedel *et al.*, 2007).

4.1.8.3. Mitochondrial membrane potential analysis

GSH pre-treatment of HaCaT cells increased mitochondrial mass (cell density independent) and reduced MMP (Figure 3.15). These data suggest that cell density microenvironment may regulate HaCaT mitochondrial biogenesis and ROS detoxification in response to SSR stress. The decrease in mitochondrial mass was transient as it recovered to control levels 24 hours post SSR. ROS production and MMP remained decreased up to 48 hours post SSR in both LD and HD HaCaT cell 123 populations (Figures 3.16B and 3.16C). These observations suggest that an increase in the total organelle number may have been accompanied by a decrease in ROS in HD cells (Figure 3.16B), suggesting increased ROS detoxification associated with increased mitochondrial number, as previously observed (Lee *et al.*, 2002).

The mitochondrial mass increase in LD cells was in parallel with a decrease in MMP and unchanged ROS levels (Figure 3.15). High MMP has been reported to increase ROS formation and to inhibit cellular respiration (respiratory control) (Lee *et al.*, 2002). In addition, mitochondrial metabolic activity may interfere with cellular oxidative stress as exemplified by the cytoprotective and ROS sensitizing effect of inhibiting the Tricarboxilic Acids Cycle (TCA) and pentose phosphate pathway respectively (LeGoffe *et al.*, 1999; Lee *et al.*, 2001; Ying *et al.*, 2002;).

Much remains to be understood about how cells perceive change in their microenvironment such as cell density and how such signals modulate MMP and ROS. It has been suggested that density dependent changes in mitochondrial specific protein expression or turnover may be involved in the process (Limoli *et al.*, 2004). In line with this suggestion is the observation by Limonelli *et al.* (Limoli *et al.*, 2004) that mitochondrial MnSOD is regulated by cell density, providing one possible mechanism for microenvironment modulation of mitochondrial ROS.

This study demonstrates that cell density and adequate control of ROS production and scavenging may be key factors in evaluating cell-type specific biological effects

of solar radiation-induced oxidative stress. Conclusions drawn from single cell density investigations should be carefully considered as they may not represent the broader spectrum of cellular responses to solar radiation irradiation.

4.1.9. Multiple Simulated Solar radiation Irradiations

The purpose of Experimental Design 3 (Table 2.1) was to determine the effect of multiple sub-lethal SSR on cell viability, metabolic activity, protein levels, mitochondrial mass and mtDNA damage and content in HaCaT cells.

The solar radiation effects on cellular apoptosis and burnt cell formation have been extensively studied (for reviews please see Bayerl *et al.*, 1995 and Pourzand and Tyrrell, 1999). A some what overlooked effect of solar radiation is the induction of cell proliferation which may have important consequences in the tumour progression associated with solar radiation over-exposure (Thomas *et al.*, 1997).

4.1.9.1. Cellular Viability

In the present study it was observed that two repeat exposures to 0.5 J/cm² SSR induced a 40% increase in HaCaT cell viability, 24 hours after the last irradiation (Figure 3.18A).

A regime of irradiations including 12 hours intervals, as the one used in the present study, can potentially shift the cellular daylight periods, also known as cellular circadian rhythm (Stevens and Rea, 2001; Kawara *et al.*, 2002) and

possibly alter steady state mtDNA repair and mitochondrial mass control. Circadian rhythm maintenance has also been linked with mitochondrial activity control in *in vitro* studies (Hardeland *et al.*, 2003; Liu *et al.*, 2007).

Another possible explanation for the observed increase in cell viability post SSR is the cellular adaptation known as hormesis, a phenomenon where low dose of an otherwise harmful stressor agent induces repair pathways and stress-response gene expression (Arumugam *et al.*, 2006; Rattan and Ali, 2007). Similarly, heat stress induced by 1 weekly hour of exposure to 41°C has been shown to improve cellular resistance to UVB and to increase the levels of antioxidant enzymes in normal human fibroblasts and keratinocytes (Rattan and Ali, 2007).

4.1.9.2. Protein Content

A consistent decrease in the total protein content of HaCaT cells (approximately 40%) was observed 7 days post irradiation (Figure 3.17B). However, the decrease in protein content was not paralleled with a decrease in cell viability at the same time point (Figure 3.17A). Increase in protein content may be due, amongst other expression up-regulation or reduction in protein gene causes. to recycling/degradation (Zhou et al., 1998; Scott et al., 2002). The former requires concomitant increase in gene expression, mRNA stability and protein translation (West et al., 1989; Piechota et al., 2006). The latter, however, is associated with lysosome damage and decreased autophagy and proteassome activity (Johnson and Daniels, 1969; Wolff et al., 1986; Boudjelal et al., 2000).

Indeed, UVR has been described to cause proteasome and lysosome damage, as well as to reduce mRNA stability (Tapryal *et al.*, 2009; Wang *et al.*, 2010). The underlying cause of protein content increase in HaCaT cells subjected to multiple SSR remains to be elucidated.

Only 2.5 J/cm² cumulative irradiations of cells presented a long term (7 days post irradiation) increase in mitochondrial mass (70%) paralleled with an increase in metabolic activity (60%) suggesting that SSR induced an overall increase in cellular metabolism in HaCaT cells (Figures 3.18B and 3.18C). In accordance with these observations, Larsson *et al.*, 2005 observed that UVA irradiation induced altered redox balance affecting cell membrane, cell cycle and rate of apoptosis in human keratinocytes (Larsson, 2005).

4.1.9.3. Metabolic Activity

The Biolog Redox dye, a tetrazolium salt derivate, was used to measure metabolic activity post SSR. In mammalian cells, the formation of coloured formazan compounds by reduction of incolor tetrazoilum salts has been shown to be mainly effected by NADH production (Stellmach and Severin, 1987). The main cellular NADH source is the mitochondrial tricarboxilic acids cycle (TCA) (Hyslop *et al.*, 1988; Przybyla-Zawislak *et al.*, 1999). However, tetrazolium salt reduction has also been observed within the cytoplasm and by non-mitochondrial membranes including the endome/lysosome compartment and the plasma membrane (Liu *et al.*, 1997).

In the present study, it was observed that SSR induced changes in mitochondrial mass that correlated to an increase in metabolic activity, as a 70% increase in mitochondrial mass was paralleled by a 50% increase in formazan production in the 2.5 J/cm² irradiated cells, 7 days post irradiations (Figures 3.17C and 3.17D). Additionally, 4 days post irradiation, a 20% decrease in metabolic activity was paralleled by an almost 40% decrease in mitochondrial mass for all the SSR up to 2.0 J/cm² (Figures 3.17C and 3.17D).

4.1.9.4. Mitochondrial Mass

It has been reported in a variety of animal models that mitochondria represent a primary site of UVR phototoxicity (Shea *et al.*, 1986; Ouedraogo *et al.*, 2000). UVR and ROS formation have been shown to cause mitochondrial morphological changes in ARPE-19 cells (Bantseev *et al.*, 2006). Oxidative stress has been associated with increase in mitochondrial mass per cell and mitochondrial morphology changes also (Limoli *et al.*, 2004; Lee at al, 2006). However, the molecular mechanisms responsible for excessive mitochondrial proliferation remain to be elucidated (Lee at al, 2006). The present study suggests that mitochondrial mass increase may be attributed to an over-compensatory stimulation (mitohormesis) effect in the 2.5 J/cm² irradiated cells. However, further data would be necessary to support this.

Cellular metabolism may be elevated by either increased mitochondrial activity and/or up-regulation of the total mitochondrial mass by biogenesis induction (Wilson-Fritch *et al.*, 2003). Increase in mitochondrial mass is usually accompanied 128 by a concomitant increase in total mtDNA content which has been suggested to be 'sensed' and balanced by total mitochondrial number instead of by relative genome copy number (Tang *et al.*, 2000).

4.2. DNA analysis

4.2.1. Analysis of mtDNA deletion

Experimental Design 3 assessed whether multiple low doses SSR elicited mtDNA deletions as such mitochondrial genome rearrangements have been suggested to play an important role in skin tumourigenesis and ROS-induced photo-ageing (Lee *et al.*, 1997; Ozawa, 1997; Birch-Machin *et al.*, 1998).

The most frequently studied mtDNA mutation in skin is a large scale deletion referred to as the "common" deletion (mtDNA⁴⁹⁷⁷) (Pang *et al.*, 1994). mtDNA⁴⁹⁷⁷ has been associated with the decline of mitochondrial respiratory functions and increase in both lipid peroxidation and antioxidant (Mn-SOD) levels (Yen *et al.*, 1994).

An association between increased mtDNA⁴⁹⁷⁷ and increased mitochondrial mass and relative content of mitochondrial genome was observed in cybrid cells in vitro (Wei *et al.*, 2001). The authors concluded that increase in mitochondrial mass and mtDNA content are associated with impaired respiratory function and enhanced oxidative stress in human cells (Wei *et al.*, 2001).

More recently, it has been suggested that another mitochondrial deletion, mtDNA³⁸⁹⁵ is generated by frequent solar irradiation exposure and has been proposed as a more sensitive marker of cumulative sun exposure than the mtDNA⁴⁹⁷⁷ common deletion (Krishnan *et al.*, 2004; Harbottle and Birch-Machin, 2006).

It has been observed that mtDNA⁴⁹⁷⁷ and mtDNA³⁸⁹⁵ deletions were detectable in basal levels in sham-irradiated HaCaT cells (Figure 3.18). These observations suggest that the mtDNA mutations may have been caused by previous exposure of the HaCaT cell line to either oxidative stress and/or solar irradiation. Indeed, previous UVR/solar radiation exposure cannot be excluded once HaCaT cells have been originally isolated from adult human skin (Boukamp *et al.*, 1988). Moreover, ROS induced by *in vitro* oxidation may also account for some degree of mtDNA deletions accumulation as it has been reported that cell culture media can be oxidized by light exposure from conventional lamps in the presence of riboflavin, a common media component (Grzelak *et al.*, 2001).

Another observation from the present study was the decrease in mtDNA⁴⁹⁷⁷ associated with increase in cellular redox and mitochondrial mass 24 hours post 2.5 J/cm² SSR (Figure 3.18). Additionally, a reduction in the mtDNA⁴⁹⁷⁷ and mtDNA³⁸⁹⁵ in HaCaT cells irradiated with 0.5 and 1.0 J/cm² SSR was observed. A pronounced induction of mtDNA⁴⁹⁷⁷ in human keratinocytes was only observed post triple exposure to 2 J/cm² irradiation using an UVA source (Koch *et al.*, 2001), similar to observations in the present study. The same authors also reported a

decrease in mtDNA⁴⁹⁷⁷ during in vitro cultivation of primary human keratinocytes (Koch *et al.*, 2001).

In the present study, a decrease in mtDNA⁴⁹⁷⁷ (Figure 3.18) was found to correlate with a significant increase in cell viability measured by the Alamar Blue® Assay (Figure 3.17A) performed 24 hours post total dose irradiation of 1.0 J/cm² (twice irradiated cells). A decrease in mtDNA⁴⁹⁷⁷ has been described in association with regeneration of respiratory activity in human fibroblasts (Bourgeron *et al.*, 1993). In contradiction, rapidly dividing or differentiating cells have been shown to be more susceptible to mtDNA deletions (Cortopassi *et al.*, 1992; Miquel, 1992; Berneburg *et al.*, 1999).

It was observed that mtDNA³⁸⁹⁵ was increased in cells irradiated with 1.0 J/cm² or higher, the same SSR dose that was found to increase cell viability, mitochondrial mass and mtDNA relative content measured by ND5/CF ratio. Whether or not the mtDNA³⁸⁹⁵ deletion is directly associated with total mtDNA up-regulation or increased cell viability could not be answered with the data collected in the present study and warrants further investigation.

Finally, present observations are in accordance with previous investigations of mtDNA⁴⁹⁷⁷ and mtDNA³⁸⁹⁵ in Non-Melanoma Skin Cancer (NMSC) samples which showed a comparatively greater number of mtDNA³⁸⁹⁵ deletions in frequently sun-exposed human skin (Krishnan *et al.*, 2004; Harbottle and Birch-Machin, 2006).

4.2.3. Mitochondrial Genome Frequency analysis by qRT-PCR

In the present study it has been observed that the mtDNA frequency, measured by the ND5 mitochondrial gene compared against the single copy nuclear gene cystic fibrosis, as previously described (Wong and Cortopassi, 2008), correlated with the percentage of mitochondrial mass changes in HaCaT cells post SSR (Figures 3.19B). Our observations are in line with previous reports that total mitochondrial mass, but not the copy number of mtDNA, is constant among cells harbouring wild-type, deleted and partially duplicated mtDNAs (Tang *et al.*, 2000).

Previous studies have also demonstrated that measuring mtDNA content provides greater sensitivity in detecting mitochondrial toxicity induced by certain drugs than cell viability or mitochondrial ultrastructural changes (Medina *et al.*, 1994). Additionally, mitochondrial proliferation induction has been reported in parallel with depletion of mtDNA induced by AZT treatment in vitro (Kiyomoto *et al.*, 2008).

More importantly, depletion of mtDNA has been associated with a reduction in tumourigenic phenotype (Israel and Schaeffer, 1987; Cavalla and Schiffer, 1997; Hayashi *et al.*, 1991), indicating that mitochondrially encoded genes are essential for the maintenance of tumoral cells (Fantin *et al.*, 2006).

Interestingly, it has been observed that mtDNA/nDNA ratio was changed if two different regions of the mitochondrial genome were quantified by RT-PCR, namely the Cytochrome b and ND5 regions. These observations suggest that the SSR-

induced impact in the mtDNA is not homogenously distributed along the entire mitochondrial genome.

Cytochrome *b* is the main component of respiratory chain complex III, being also the only cytochrome coded by mtDNA (Esposti *et al.*, 1993). Cytochrome *b* has an important role in cellular respiration and ROS production and its expression has been reported to be regulated by cellular activity in neurons (Kaminska *et al.*, 1997) and that inhibition of cytochrome *b* oxidation generates mitochondrial H_2O_2 and O_2 (Neale *et al.*, 1993; Senoo-Matsuda *et al.*, 2001). Moreover, mutations in cytochrome *b* have been reported to result in exercise intolerance in humans (Blakely *et al.*, 2005).

In the present study it was observed that cytochrome *b* gene quantification by RT-PCR directly correlated with mitochondrial mass in HaCaT cells irradiated with a cumulative SSR dose of 1.5 J/cm² (Figure 3.19D). Higher SSR doses (2.0 and 2.5 J/cm²) clearly induced an inversely proportional correlation between ND5 and cellular metabolism (Figure 3.19C).

In accordance with present study observation, it has been reported that increased mitochondrial number, Cyt b gene copy number and Cyt b mRNA expression were observed in the cardiac muscle cells of a murine model of chronic type 1 diabetes, in which oxidative stress was found to be coincident with increase in mitochondrial biogenesis (Franko *et al.*, 2008). Based on the present observations, it has been hypothesised that Cyt b region relative quantification may be indirectly

associated with the mitochondrial mass control (mitochondrial biogenesis) post SSR stress.

4.2.4. Mitochondrial Gene Copy Number Variation

The ND5 region encodes for the NADH dehydrogenase 5 which is active at complex I of the mitochondrial respiratory chain. In the present study it has been observed that ND5 quantification by RT-PCR correlated with relative metabolic activity measured by the Biolog Redox assay (Figure 3.19C). It has been reported that cellular respiration is tightly regulated by ND5 gene expression (Yidong *et al.*, 2000). In mitochondria, transcription is essential for mtDNA replication as it requires an RNA primer that is generated from the L-strand promoter (Schinkel and Tabak, 1989; Tracy and Stern, 1995). It seems possible that the observed increase in ND5 quantification may suggest a concomitant increase in the ND5 protein production and cellular respiration, as indirectly inferred by the increase in metabolic activity measured by the Biolog Redox assay (Figure 3.19C). No less importantly, the ND5 gene is within the mtDNA region that is lost when a "common" deletion occurs (Schon *et al.*, 1989) and as so, it would be expected to be found in a reduced ratio in cells subjected to oxidative stress and containing increased mtDNA⁴⁹⁷⁷ frequency.

Intriguingly, ND5/CF ratio was found to be increased in cells post 2.0 J/cm² SSR, in which an increase in mtDNA⁴⁹⁷⁷ was also observed (Figures 3.19B and 3.18). Homoplasmic frame-shift mutation in the ND5 gene was recently found to be associated with increased tumour-forming capacity in *in vitro* and *in vivo* models 134 (Park *et al.*, 2009). Alterations in OXPHOS resulting from mitochondrial dysfunction have long been thought to be involved in tumourigenesis (van Waveren *et al.*, 2006). Although confirmation of present study correlations using independent methods are warranted (i.e. southern blot), the present observations still suggest that mtDNA replication, and possibly expression, is heterogeneously impaired by multiple sub-lethal SSR exposures.

Moreover, unbalanced quantification of the two disparate mtDNA regions may have been caused by SSR-induced changes in the mitochondrial genome replication, as the organization of mtDNA is strongly related with its replication (Clayton, 1991). However, the actual mtDNA replication process is not completely known and up to now, two main mtDNA replication models are proposed. The first one, known as the strand-assymetric model, suggests that replication is unidirectional, starting from the origin of replication located on the heavy strand (OH) (Shadel and Clayton, 1997; Moraes, 2001; Nishigaki *et al.*, 2004). The second model, called the 'strand-coupled model', proposed that mtDNA is synthesized from multiple, bidirectional origins (Holt *et al.*, 2000; Young *et al.* 2002; Bowmaker *et al.* 2003).

Given that ND5 is located downstream from Cyt b, which is located nearest to the OH, both mtDNA regions would be expected to be transcribed in tandem and as so, to maintain their relative proportions relatively constant overtime. The present study observations, in which different amounts of Cyt b and ND5 were quantified by RT-PCR, favour the 'strand-coupled model', which proposed that mtDNA may be

synthesized from multiple, bidirectional origins. The reason for these disparate mtDNA/nDNA quantification observations depending on the mtDNA region analysed is currently unknown and warrants further investigation by an independent approach.

mtDNA regions next to and including the D-loop (including gene regions IS and ND5) are a hotspot for rearrangement break-points. It has been described that one such hotspot is adjacent to a protein binding site and it has also been suggested that mtDNA recombination may be facilitated by protein-protein interaction (Kajander *et al.*, 2000). Additionally, UVR is long known to induce protein-protein and protein-DNA cross-links (Sutherland, 1978; Lai *et al.*, 1987; Peak and Peak, 1991) what may be a possible cause of solar radiation-induced mtDNA damage.

4.2.5. Oxidative mitochondrial DNA damage and repair assay

Experimental Design 4 aimed to determine the induction and repair of mtDNA damage in HaCaT cells following SSR exposure. In the course of this study it was unexpectedly observed that nDNA also showed SSR-induced damage. Additionally, in the present study, the mtDNA/nDNA oscillations and the mtDNA³⁸⁹⁵ deletion accumulation post dose-curve and time lapse studies have also been analysed. Using QPCR assay the induction and repair of lesions were determined in an 8.9 Kb mtDNA region and a13.5 Kb nDNA region of the DNA polymerase β gene.

Data obtained in this study show: (1) SSR exposure increased mtDNA/nDNA; (2) HaCaT cells incurred mtDNA and nDNA damage post SSR; (3) decrease in the ND5 136 amount post SSR, possibly indicative of increased mtDNA deletions frequency on the mtDNA major arc region; (4) mtDNA³⁸⁹⁵ deletion frequency was decreased post low dose SSR and increased post higher dose SSR, as early as 15 minutes post SSR; (5) mtDNA lesion accumulation and mitochondrial mass reduction occurred in parallel at 3 hours post SSR.

HaCaT cells responded to SSR-stress by elevating the mtDNA/nDNA ratio, as early as 30 minutes post a single 5.0 J/cm2 irradiation. Increase in energy production may be achieved by an increase in mitochondrial biogenesis (Civitarese *et al.*, 2007) and/or an up-regulation in mitochondrial gene expression post UVR (Vogt *et al.*, 1997; Ashida *et al.*, 2003; D'Souza *et al.*, 2007). Additionally, mtDNA replication is relatively independent of cell cycle, as there is no requirement for each molecule to replicate over a cell-cycle (Clayton, 1996).

The control of mtDNA number is a process not entirely described despite an increasing number of publications on the subject (MacAlpine *et al.*, 2000; Moraes, 2001). Moreover, in accordance with findings from the present study, increased mtDNA levels are usually described in association with increased mitochondria number (Ekstrand *et al.*, 2004), although changes in organelle number and mitochondrial genome content are not always found to be directly associated (Ozawa,1997; Higuchi, 2007). It remains to be fully described how the nucleus senses mtDNA levels and regulates the gene expression of genes associated with mtDNA replication machinery, as the transcripts for mtDNA pol gamma, mitochondrial RNA polymerase and mitochondrial transcription factor A (mtTFA)

(Chinnery and Samuels, 1999; Moraes, 2001; Kanki *et al.*, 2004). Furthermore, the 'limiting factors' theory states that mtDNA levels stop increasing when replication factors necessary for its replication are committed or consumed during replication of existing molecules (Tengan *et al.*, 1997).

As oxidative damage and repair analysis was based upon differences in LR-PCR product it was important to evaluate for mtDNA frequency. Real time quantification of a short conserved mtDNA fragment (IS) normalised against CF provided a measure of undamaged mtDNA frequency, given the low probability of introducing lesions in such small DNA fragments in comparison to longer amplicons (Santos *et al.*, 2006).

The QPCR assay employed, is based on the principle that oxidative DNA lesions inhibit DNA polymerases (Kovalenko and Santos, 2009) and that DNA lesions such as strand breaks, oxidative damage, and bulky DNA adducts, will slow down or block the progression of DNA polymerase (Santos *et al.*, 2006). Thus, an increased frequency of DNA lesions corresponds to decreased DNA amplification. Using this technique it was found that SSR increased mitochondrial and nDNA damage accumulation, relative to sham-irradiated control cells.

The frequency of DNA lesions was initially observed to increase post SSR mtDNA and nDNA though changes were not linear with SSR dose (Figure 3.21). Previous studies have shown that mtDNA is more prone to UVR-induced oxidative damage

than nDNA (Allen and Coombs, 1980; Backer and Weinstein, 1980; Niranjan *et al.*, 1982; Balansky *et al.*, 1996; Olivero *et al.*, 1997; Richter et al., 1998).

Consistent with these results, in the present study, it was observed that mtDNA lesion accumulation peaked 3 hours post 5.0 J/cm² SSR and that the damage was partially repaired 6 hours post irradiations. It has been demonstrated that over-expression of DNA repair enzymes in mitochondria leads to increased mtDNA repair following oxidative damage, resulting in increased cellular survival (Dobson *et al.*, 2000; Rachek *et al.*, 2002; Druzhyna *et al.*, 2008). Accumulation of large deletions in mtDNA in postmitotic tissues and abnormal organelle proliferation could lead to an increase in mtDNA replication rates (Johnston *et al.*, 1995; Moslemi *et al.*, 1996). Furthermore, mtDNA damage can be regulated by growth factors and altered transcription factor activity (Goyns, 2002; Suematsu *et al.*, 2003).

In accordance with present study observations, Godley *et al.*, (2005), reported that human retinal pigment cells exposed to blue light (390-550 nm) with a 2.8 mW/cm² irradiance, for up to 9 hours, presented with mtDNA damage that peaked at exactly 3 hours post irradiation. However, the authors found a higher lesion content (70 lesions per 10 Kb) in comparison with our findings (0.15 lesions per 10 Kb). There were though, profound differences between the present experimental design and those of Godley *et al.*, 2005, including but not limited to: irradiation source – (SSR vs blue light); dosimetry (maximum dose included in our study was 7.5 J/cm²); cell type (keratinocytes vs human retinal pigment cells); cell

line-specific repair mechanisms; These can easily account for the quantitative differences in mtDNA lesions observed between studies.

However, it is remarkable that the time-lapse of mtDNA damage and repair expression was still similar between both studies, ie, mtDNA damage observed at 3 hours post SSR and blue light irradiation as well as mtDNA repair occurring at 6 hours gap post both visible and blue light-induced damages, suggesting a possible ciclicity in mtDNA repair/damage accumulation.

nDNA damage is expected to be generated by direct DNA strand breaks or by long lived ROS able to reach the nucleus (Sedgwick, 1976; Berneburg *et al.*, 1997; Halliday, 2005). In the present study, nDNA lesion frequency peaked at 48 hours post exposure to 5 J/cm² SSR. (Figure 3.22D). Interestingly, 24 hrs post SSR, mtDNA and nDNA lesions numbers were comparable, suggesting that some shared DNA repair mechanisms may be following circardian rhythm control re-set clues (Sancar *et al.*, 2000; Bolige *et al.*, 2005; Unsal-Kacmaz *et al.*, 2005; Kondratov *et al.*, 2007; Collis and Boulton, 2007; Kang *et al.*, 2009). Moreover, HaCaT cells are known p53 mutants (Lehman *et al.*, 1993) and p53 has been found to be associated with increase in DNA damage accumulation (Cox and Lane, 1995; Vafa *et al.*, 2002) which may interfere with normal nDNA repair mechanisms (Yang *et al.*, 2006; Ferguson-yates *et al.*, 2008).

mtDNA is more susceptible to damage as: (1) it is closely located to ROS generation sites (ETC), (2) it lacks histone protection, (3) mitochondria contain a number of

chromophores which absorb light in the visible region (flavins and cytochromes) (Allen and Coombs, 1980; Backer and Weinstein, 1980; Niranjan *et al.*, 1982; Shay and Werbin, 1987; Bandy and Davison, 1990; Balansky *et al.*, 1996; Olivero *et al.*, 1997; Richter *et al.*, 1998). mtDNA has recently been reported to be sheathed by TFAM in much the same way as histones cover nDNA (Nils-Goran *et al.*, 1998) and organized into discrete structures called nucleoids (Satoh and Kuroiwa, 1991; Garrido *et al.*, 2003), in which a number of antioxidants and repair proteins have been reported to be localized (Miyakawa wt al, 1987; Meeusen *et al.*, 1999; Kaufman *et al.*, 2000; Bogenhagen *et al.*, 2003; Garrido *et al.*, 2003).

The QPCR analysis, although reliable, does not define the specific type of lesion present on the DNA post SSR (Kovalenko and Santos, 2009). Additionally, whether HaCaT cell mtDNA repair mechanisms are similar to that of normal primary human keratinocytes remains to be further investigated.

It is worth mentioning that melatonin, a major neuro-endocrine regulator exerts potent antioxidant and anti-aging effects on mammalian skin (Reiter, 1995; Kotler *et al.*, 1998). Recently it has been shown that human keratinocytes are sites of extrapineal melatonin synthesis (Fisher *et al.*, 2006). Moreover, melatonin is classically considered as a hormone that regulates the circadian day-night rhythm (Lewy *et al.*, 1984; Brzezinski, 1997). More importantly, melatonin has been shown to decrease the UV-induced DNA fragmentation and cellular stress and to maintain MMP and to reduce immunological response signalling (caspases 9, 3 and 7 production) post UVR in keratinocytes (Fisher *et al.*, 2008).

A circadian rhythm-associated mechanism of DNA repair may be inferred due to the 24 hours reduction in both mitochondrial and nDNA damage observed in the present study. Whether the mtDNA repair post SSR observed in this study may be attributed to melatonin production by HaCaT cells *in vitro* conditions remains to be further elucidated.

The present study aimed to evaluate the possible asymmetrical amplification of the major and minor arcs of mtDNA and in so doing, provide an indirect measurement of SSR-induced deletion accumulation in HaCaT cells. The ND1 gene is located in the mtDNA minor arc region, which has a lower deletion rate than the major arc (Ruiz-Pesini *et al.*, 2007). The ND5 gene is located in the mtDNA region affected by several characterised deletions, and is within the major arc (Ruiz-Pesini *et al.*, 2007).

The present study finding, that ND1 gene copy number was not proportional to ND5 genes copy number may be due to: (1) induction of mtDNA deletions in the major arc leading to a reduction in the ND5 gene quantification; (2) induction of bidirectional mtDNA replication allowing for the ND1 gene to be replicated independently of ND5; 3) a combination of (1) and (2). Further investigations are warranted to determine the mechanisms by which SSR disrupts ND1/ND5 ratio, mtDNA replication, mtDNA damage and repair.

Two main models for the replication of mtDNA are considered. In the first one, the asymmetric mtDNA replication model proposes that replication is initiated unidirectionally from the heavy strand origin in the D-loop region so that the parental heavy strand is progressively replicated (Cyt b and ND5 located downstream to OH and supposedly, replicated first then ND1) until the light strand synthesis is initiated further downstream (nearest to ND1) (Clayton, 1982; Shadel and Clayton, 1997; Clayton, 2003). A second mtDNA replication model supports the hypothesis that a bi-directional strand replication may occur instead (Holt *et al.*, 2000; Yasukawa *et al.*, 2005).

4.2.6. Quantification of mtDNA³⁸⁹⁵

To more sensitively determine if SSR induced a specific mtDNA deletion, recently associated with highly solar radiation exposed human skin, mtDNA³⁸⁹⁵ was quantified using a Taqman assay. No correlation between the level of mtDNA³⁸⁹⁵ and the number of mtDNA molecules or damaged mtDNA content was observed. This finding is of particular interest as it suggests that mtDNA³⁸⁹⁵ formation, mtDNA content regulation and mtDNA oxidative stress damage may arise via different mechanisms.

SSR radiation induced a biphasic dose-response in Δ mtDNA³⁸⁹⁵ accumulation, where lower dose SSR induced a decrease in mtDNA³⁸⁹⁵ ratio whereas higher dose SSR induced a modest increase in the deleted mtDNA relative to the shamirradiated controls. Additionally, 24 hours post irradiation the mtDNA³⁸⁹⁵ frequency was comparable to sham-irradiated controls (Figure 3.24).

The induction of mtDNA³⁸⁹⁵ was found to be the highest in HaCaT cells at 6 hours post 5.0 J/cm² irradiation concomitantly with the first observation of mtDNA oxidative damage repair (Figures 3.22D and 3.23B). Accumulation of large deletions in mtDNA in postmitotic tissues and abnormal organelle proliferation could lead to an increase in mtDNA replication rates (Diaz *et al.*, 2002). Indeed, in the present study a paralleled increase in mtDNA/nDNA ratio and a decrease in the Δ mtDNA³⁸⁹⁵ ratio have been observed only post lower SSR doses (Figure 3.19B (IS/CF) and 3.24A).

As mtDNA is known to segregate between daughter cells in mtDNA copy numberdependent manner (Clayton, 1996), it may be possible that increased mtDNA levels would be associated with decrease in deleted mtDNA molecules, possibly as a way of maintaining the supply of high cellular energetic demands induced by SSR-stress.

4.2.5.1. Mitochondrial DNA lesions and mitochondrial mass correlation Interestingly, mitochondrial mass and mtDNA lesions accumulation oscillated following similar patterns up to 1 hours post a single 5.0 J/cm² irradiation dose (Figure 3.23B). However, 3 hours post irradiation, an increase in mtDNA lesions was observed in parallel with a 40% decrease in mitochondrial mass (Figure 3.23B). These results suggest the existence of a regulation between mtDNA damage and mitochondrial mass control, and that possibly a co-regulation of mitochondrial quality control by mitochondrial biogenesis; mitochondrial 144 dynamics (fusion and fission) and mitophagy may be induced by solar radiation. More recently, new characteristics of mitochondrial morphology dynamics changes and mtDNA organisation in nucleoids have shed new light in to the complexity of mtDNA stability and expression (Santel and Fuller, 2001; Karbowski and Youle, 2003; Legros *et al.*, 2004).

4.3. Mitophagy Analysis

In the Experimental Design 5 the mitochondrial and lysosomal fluorescent costaining was used as an indicator for mitophagy induction in A375 melanoma cells post SSR. Autophagy is responsible for degradation and turnover of cell organelles, including mitochondria (Kim *et al.*, 2007). Autophagy induction is characterized by increased proliferation of autophagosomes and autolysosomes, protein degradation and catabolic energy production (Plomp *et al.*, 1987). LTG is an acidotropic dye that primarily detects lysosomes (Jiang *et al.*, 1990). Subsequent fusion of a non-acidic autophagosome compartment with a lysosome compartment generates an acidic autolysosome (Klionsky *et al.* 2008). MTR stains mitochondria in live cells and its accumulation is dependent on membrane potential (Gilmore and Wilson, 1999).

A Q-Sun solar simulator (Q-Panel, FL) was employed to deliver SSR which is superior to the use of a fluorescent lamp as it does not include the small but significant portion of photons in the UVC nor does it over-represent the UVB spectrum (43% in fluorescent lamps as opposed to only 4% for solar radiation) (Gasparro and Brown, 2000).

To examine the effects of SSR exposure on cell size, confocal images were analyzed using the Image Processing Kit 4.0.25 software (Zeiss). A minimum of 50 cells were analyzed per treatment and double nucleated cells (indicative of mitotic or polyploid cells) were not included as a quality control for cell cycle-associated differences in cell size. Cells were observed to be reduced in size to approximately 75% that of control following all doses studied (Figure 3.25) indicating that SSR significantly affects cell size regulation in A375 cells.

Cell volume change is an important signaling mechanism associated with changes in the rate of oxidative metabolism, solute transport, initiation of apoptosis, regulation of cell cycle and hormone release (Klein, 2000; Lang, 2007). Cell swelling and shrinking is typically associated with increased and decreased macromolecule synthesis such as glycogen, lipids and proteins and is also influenced by the cell's hydration status (Häussinger *et al.*, 1994). In addition, UV light induces p53-dependent cell cycle arrest which is thought to be required for efficient repair of DNA lesions and results in cell size increase, (Al-Mohanna, 2001). A possible correlation between cell size regulation and both lysosomal and mitochondrial number homeostasis may be inferred from the observation that increased cell area and lysosome to mitochondria ratio was observed post SSR in cells that were glutamine starved and in cells that were supplemented with FCCP and creatine.

4.3.1. Lysosomes Quantification

The number of LTR-labeled organelles per A375 cell correlated with increasing SSR doses, and total lysosome number also showed a dose-dependent increase (Figure 3.25B). Specifically, the number of small sized (< 1 μ m²) acidic organelles per A375 cell increased 6-8 fold in cells irradiated with 0.25, 0.5 to 2.5 J/cm², indicating a possible up-regulation of lysosome biogenesis post SSR-induced cell stress.

SSR induced small lysosome proliferation in a direct dose-dependent manner in A375 cells. The observed increase in small lysosomes without any increase in mitophagy indicates a multi-functional nature of the lysosomal population which includes cytoplasmic protein autophagy (Kristensen, 2008). In general, increases in both lysosome size and number are frequently observed and, when autophagosome-lysosome fusion is blocked, larger autophagosomes are detected, possibly due to autophagosome-autophagosome fusion (Bains *et al.*, 2009).

4.3.2. Mitochondrial Morphology

Post SSR exposure, a heterogeneous mitochondrial population was observed, encompassing two major distinctive populations, one brightly stained and the second being more faintly stained (with increased and decreased MMP respectively) (Figure 3.26).

Photoaging is associated with decreased activity of the tricarboxylic acid cycle, beta-oxidation, and OxPhos enzymes (Brunk and Terman, 2002). Furthermore, 147 mitochondrial fusion permits metabolic substrate diffusion, MMP recovery and maintenance of tumoural respiratory activity in hypoxic regions (Frank 2006). Mitochondrial fusion plays an important role in mitochondrial function, allowing energy production and transmission between different cellular regions (Amchenkova et al., 1988).

It has been recently reported that UV stress-induced mitochondrial hyper-fusion is associated with increased mitochondrial ATP production, which may constitute a novel adaptive pro-survival response against selective stress (Tondera et al., 2009). Earlier studies on cardiac myocytes suggested that large mitochondria are less prone to autophagy, allowing enlarged damaged organelles to accumulate with time (Terman *et al.*, 2003). This size related mitophagy escape is further supported by the observation that the formation of a large autophagosome is more energy consuming than the formation of a small one (Skulachev, 2006).

In this study, effects of SSR in combination with glutamine supplementation or depletion, pre-treatment with melanin, FCCP and creatine were evaluated, in particular for their potential to induce mitochondrial network re-organization and mitophagy. The observation that FCCP, creatine and low glutamine treated cells presented fragmented mitochondria correlated with the observation of an increase in lysosomal and mitochondrial fluorescence co-localization, indicative of increased mitophagy (Figure 3.29D).

Previous studies have demonstrated that uncoupling agents cause overall mitochondrial fragmentation, fusion arrest and increased lysosome and autophagosome content in isolated cardiomyocytes (Legros *et al.*, 2002; Ishihara *et al.*, 2003). Reduced cellular ATP levels and MMP dissipation were found to partially inhibit mitochondrial fusion, whereas FCCP, a well characterized inhibitor of MMP, is known to stimulate ROS production, ATP hydrolysis and inhibit OxPhos which ultimately culminates in mitochondrial fission, perinuclear localization and mitochondrial programmed death (Leonard and Schapira, 2000; Lyamzaev *et al.*, 2004).

Oxidative stress, above a certain threshold, can cause lysosomal rupture due to intralysosomal iron-catalyzed peroxidative reactions (Kurz *et al.*, 2008). In the present study, it was observed decreased LTG accumulation post SSR in FCCP and creatine treated cells as well as in glutamine starved cells.

Recycling of damaged mitochondria via selective lysosomal degradation is an important protective mechanism from stress-induced damage, especially important in post-mitotic cells (Yu, 2008). It has been suggested that mitochondria that overproduce ROS also demonstrate opening of their permeability transition pore (PTP) and are directed to mitophagy (Rodrigues-Hermandes *et al.*, 2009). Mitophagy is also thought to play a key role in retarding accumulation of somatic mutations of mtDNA with age (Lemasters, 2005). Besides that, age-related changes in mtDNA and function are likely to be the consequence of mitophagy failure in a small number of severely injured mitochondria (Donati, 2006).

Age-dependent decrease in mitochondrial fusion and fission activity (Tatsuta and Langer, 2008) further highlights that the balance of mitochondrial dynamics is an important anti-ageing and possibly anti-tumoural mechanism, as damaged mitochondria and mtDNA accumulation has been associated with photo-damaged tissues and potentially with skin cancer initiation (Terman *et al.*, 2006; Yang *et al.* 2004).

Therefore it has been explored whether mitochondrial morphology changes were associated with SSR response, metabolic energy source or onset of mitophagy in A375 cells. Sham SSR exposed and glutamine starved cells presented brightly stained mitochondria of complex morphology (Figure 3.27). Post SSR, glutamine starved A375 cells presented increased cell area and a highly fragmented mitochondrial population (Figures 3.28A and 3.27). More importantly, A375 cells supplemented with glutamine did not present cell swelling nor mitochondrial fragmentation post SSR.

The increased mitophagy and fragmented mitochondria observed in glutaminestarved and SSR exposed A375 cells (Figure 3.29D and 3.27) may be associated with TCA cycle intermediate deficiency, as glutamine in known to feed into the TCA cycle via conversion to 2-oxoglutarate (Kovacevic, 1972). TCA perturbation may have various consequences, as numerous metabolic pathways converge there. Indeed, apoptosis has observed in cells with altered TCA in which ATP levels were kept high by glycolysis (Albayrak *et al.*, 2003).

Newly synthesised glutamine is metabolized in mitochondria by phosphateactivated glutaminase, yielding glutamate and ammonia. The glutamine-derived ammonia was found to interfere with mitochondrial function, to induce excessive ROS formation and MTP, culminating in cellular dysfunction and cell swelling in astrocytes (Albrecht, 2006).

Glutamine metabolism has been described as crucial for tumour cell metabolism, as some cancer cells were found to survive glucose starvation if glutamine and nucleotides were available whereas, other cancer types presented a mandatory requirement for glutamine or its metabolites for survival (Wice *et al.*, 1981; Medina, 2001). Additionally, levels of glutamine/glutamate were found to be higher in primary and secondary melanoma biopsies relative to normal and benign tissue (Bourne, 2005).

Increased mitochondrial fusion (Figure 3.27) has been observed in A375 cells glutamine supplemented post SSR. It has been reported that glutamine-induced augmentation of glutathione levels may potentially increase ROS detoxification, which in turn may affect the decreased mitophagy observed in glutamine supplemented and solar radiation irradiated A375 cells (Figure 3.29D) (Fläring *et al.*, 2003; Curi *et al.*, 2005). In addition, glutathione has been reported to play an important role in mitophagy regulation in yeast (Deffieu *et al.*, 2009).

The influence of creatine supplementation on energy supply in melanoma cells post SSR has not been described in detail yet. In the present study, the effects of creatine supplementation on mitochondrial dynamics and mitophagy induction post SSR in A375 melanoma cells were investigated.

Creatine supplementation in sham SSR-exposed cells induced the formation of highly fragmented mitochondria, including round, enlarged and swollen mitochondria and diffuse cytoplasmic MTR staining (Figure 3.27). Creatinestimulated respiration is associated with intra-mitochondrial creatine kinase activity, which increases mitochondrial inter-membrane ATP/ADP ratios, and produces phosphocreatine by ATP consumption in muscles (Kuznetsov *et al.*, 1996).

In human epidermal cells, stress-induced mitochondrial kinase activity reduction is associated with depleted mitochondrial energy supply whereas creatine supplementation in human keratinocytes increases mitochondrial function and protects against ROS stress and UV damage *in vivo* and *in vitro* (Lenz, 2005). Normal human skin expresses cytosolic and mitochondrial creatine kinases as well as mitochondrial creatine transporters (Schlattner *et al.*, 2002).

ROS formation has been described as a possible cause for reduced creatine kinase activity in aged skin (Dolder *et al.*, 2001). Furthermore, an analogue of creatine (cyclocreatine) was found to inhibit 80-90% of human melanoma cell mobility in vitro (Mulvaney *et al.*, 1998) indicating a possible negative effect on melanoma

cells. Further studies are necessary to elucidate the creatine mechanism of action in A375 cell mitochondrial dynamics.

Melanin is known to act as a free radicals scavenger and is important in protecting pigmented cells from natural toxins and ROS accumulation (Rozanowska, 1999). In the present study, melanin supplementation was found to induce mitochondrial fusion and/or prevent fission in A375 melanoma cells post SSR. It was observed that melanin supplementation maintained the lysosome / mitochondria population balance (Figure 3.28D) and prevented mitophagy in melanoma cells post SSR.

Many recent reports show evidence of an important association between mitochondria and melanin synthesis. In melanocytes, it is known that UVR and metabolic inhibitors induce a stress response pathway, which then alters the levels of tyrosinase, a key enzyme in the melanin biosynthesis pathway, inducing melanin synthesis (Ramirez-Bosca *et al.*, 1992; Busca and Ballotti, 2000). In non-melanocytes, UVR affects mitochondrial function and apoptotic signaling pathways (Hakem *et al.*, 1998). Moreover, inhibitors of the mitochondrial FO/F1 ATPase were found to induce pigmentation in melanocytes, further indicating a role for mitochondrial signal transduction in the induction of melanin production (Williams *et al.*, 2004).

4.3.3. Co-localisation Analysis

Figure 3.28 illustrates that the lysosome to mitochondria ratio correlated with the relative co-localization fluorescence of the MTR and LTG shown in Figure 3.29D, 153

suggesting that changes in the organelles delicate balance is linked to the increase in lysosomal removal of mitochondria that have sustained damaged membranes post SSR.

Interestingly, brighter and more discrete lysosomes were observed in glutamine and melanin supplemented A375 cells (Figure 3.29A) and the lysosome to mitochondria ratio remained comparable to that in sham-irradiated cells post SSR, (Figure 3.28B). A decrease in total mitochondrial and lysosomal fluorescence was observed (Figures 3.27B and 3.28C) along with increased numbers of mitophagic events (Figure 3.29D). FCCP and creatine supplementation as well as glutamine starvation all increased SSR-induced mitophagy. It is likely that each compound contributes to mitophagy via disparate mechanisms that converge toward the same outcome of increased mitochondrial fission and decreased MMP (Figures 3.27, 3.28 and 3.29).

As measured by LTG and MTR fluorescence co-localization, glutamine and melanin supplementation inhibited mitophagy (Figure 3.29D). The glutamine and melanin supplemented cells preserved all the morphological parameters when analyzed post SSR, suggesting that energetic balance, antioxidant activity of glutamine and melanin derivatives as well as mitochondrial fusion induction may all play an important role in melanoma cell survival post SSR.

4.4. Summary

In the present study cell death, cellular DNA and protein content, metabolism and mitochondrial mass and function were studied in melanoma-derived and spontaneously immortalized human keratinocytes, exposed to sub-lethal doses of simulated solar radiation.

Cellular viability, metabolism and mitochondrial mass and membrane potential were also found to be disrupted by SSR. Lower SSR doses induced the more pronounced changes in all cellular parameters investigated. Moreover, cellular proliferation and increase in mitochondrial mass and metabolism were observed in exposed C32 melanoma-derived cell line whereas cell death by necrosis, reduction in mitochondrial mass and mitochondrial membrane depolarization were observed in exposed HaCaT and A375 cells, indicating the existence of a cell-type specific response to SSR. Such differences in cellular response to SSR possibly reflect the various mechanisms that led to cell transformation and immortalisation in the cell lines analysed.

UVR is the major environment stressor of skin cells, which have evolved adaptive mechanisms to cope with same. One important observation of the present study was the proliferative advantage conferred by SSR to C32 cells in comparison to the other cell types analysed (HaCaT and A375). Different cell lines are highly likely to have distinct energetic needs and coping mechanisms for solar radiation stress. From the present study data it seems also clear that low SSR (0.5 J/cm²) induced more pronounced changes in cell viability and mitochondrial end-points than 155 higher SSR doses. More detailed studies are necessary to establish the mechanism of cell death (e.g., necrosis, autophagy, etc.) and mitochondrial mass adaptation induced by SSR in each cell type. Knowledge of mitochondrial mass variation in response to solar radiation and its effects on antioxidants and energy production opens up possible new intervention points in anti-melanoma therapies.

Few studies have detailed specific changes in mitochondrial parameters post SSR in association with varying cell densities which can potentially influence ROS detoxifying capability and subsequent changes in cellular physiology. Tumour progression and growth factors are known to induce genetic or epigenetic changes in tumour cells and are reported to be affected by cellular microenvironment (Timmer et al., 1994; Boniver et al., 1985). Additionally, tumour masses are not constituted by homogeneous cell densities (Stoler et al., 2002), which further support the relevance of in vitro modelling of cellular population diversity, modelled in the present study. The majority of previous in vitro SSR studies analysed cells of equal or similar density (Zmijewski and Slominski, 2009; Jones et al., 2002). However, such experimental design overlooks the impact of cell density, and ultimately the cell density-associated differences in cell population aging and cell-cell communication. Although such cell density variations are not sufficient to model the complexity of skin carcinoma microenvironment it provides an indication of how microenvironment may influence mitochondrial and cellular physiology response to UVR.

Besides acting upon mitochondrial metabolism, SSR promoted an increase in cell viability, protein content and cellular metabolism in HaCaT cells. Thus, relatively low doses of SSR significantly influenced mitochondrial and cellular homeostasis in HaCaTo cells *in vitro*.

The present study results demonstrate that exposure to multiple low-dose SSR causes protein content, metabolic activity and mitochondrial mass increase and multiple mtDNA damage events. Additionally, it was observed that mtDNA Cyt b gene quantification correlated with mitochondrial mass and that ND5 gene quantification had a direct correlation with metabolic activity, up to 1.5 J/cm² irradiated cells. More importantly, higher SSR doses (2.5 J/cm²) induced an inversion between the ND5 and metabolic activities ratio which may be associated with cellular oxidative stress response mechanisms. These observations still lack a mechanistic description and have not yet, to the author's knowledge, been described in *in vivo* studies.

Therefore, human keratinocyte mitochondria are sensitive to solar radiation damage and apparently adapt, even post low-dose SSR, to the new energetic needs to maintain cellular homeostasis. Since mitochondria are responsible for energy production and a hub for multiple metabolic pathways and physiological endpoints, elucidating the impact of solar radiation at environmentally relevant doses on mitochondrial function and mtDNA, as addressed in the present study, will advance the understanding of cutaneous carcinogenesis.

In addition, another objective of the present study was to investigate the role of mtDNA damage and repair in HaCaT cells. A quantitative PCR (QPCR) assay was used to study DNA damage in sets of dose-curve and time-lapse experiments. To investigate whether mtDNA/nDNA ratio changes were associated with differential mtDNA damage repair three different mtDNA regions were quantified and normalized against a single copy nuclear gene. To elucidate some of the specific mtDNA lesions induced by SSR, the frequency of occurrence of a mtDNA deletion recently described in association with frequently solar radiation exposed skin was analysed (ΔmtDNA³⁸⁹⁵) (Krishnan *et al.*, 2004; Harbottle and Birch-Machin, 2006).

In the present study it has been found that mtDNA and nDNA were both prone to oxidative stress damage at different time points post SSR-induced stress although, as expected, mtDNA was oxidatively damaged to a greater extent than nDNA. Interestingly, HaCaT cells displayed time-specific expression of mtDNA damage and great capacity to repair SSR-induced DNA lesions.

Increased mtDNA damage may lead to mitochondrial dysfunction resulting in decreased ATP supply and further increased ROS production (Linnane *et al.*, 1989; Kowald and Kirkwood, 1993; Goodell and Cortopassi, 1998). It is possible that mitochondrial damage above a certain threshold can generate ROS and lead to a vicious cycle of damage that, even in the presence of mtDNA repair system, will result in persistent mtDNA damage. It is also possible that the mtDNA repair mechanism may be an important mechanism to cope with SSR-stress and other environmentally initiated cell damage (Abramova *et al.*, 2000).

Additionally, when cells are SSR-stressed, spatial redistribution and differences in traffic and permeability of proteins, nucleotides, transcription factors, antioxidants and energetic sources to mitochondria may be initiated. Reduction in mitochondrial transcripts and proteins associated with DNA repair and replication may also be targets of the interference of SSR. Alternatively, the SSR may alter the cellular circadian rhythm indirectly altering cellular antioxidant defences, mtDNA replication and repair dynamics. Another possible effect of mtDNA deletion accumulation is that, above a certain thereshold, the same may potentially decrease the ETC activity, reducing both energy production and the formation of potentially damaging ROS.

The vulnerability of mtDNA and nDNA to SSR strongly supports the photo-toxic effects of visible irradiation upon cellular functioning (Kagan *et al.*, 2002). Such dysfunction may be at maximum in skin which is regularly exposed to solar radiation throughout human life (Genova *et al.*, 2004). Moreover, SSR-induced damage is likely to contribute to skin cancer initiation and progression (Croteau and Bohr, 1997) and as so, is a critical factor in human health.

The present study describes mitochondrial morphology changes and mitophagy protection through glutamine and melanin supplementation post solar radiation exposure in A375 amelanotic melanoma cells. Observations from the present study also support the hypothesis that changes in mitochondrial morphology play an important role in mitophagy regulation following solar radiation exposure and that

such changes may be dependent on energetic source and antioxidant availability. An intricate correlation between form and function, observable as a continuous balance of mitochondrial biogenesis, fusion and fission, is present in "resting" conditions as well as during cellular and mitochondrial response to environmental stressors (Hom and Sheu, 2009). The precise changes in mitochondrial dynamics post solar radiation exposure in melanoma cells are not well characterized. In this study mitophagy post SSR was evaluated using confocal microscopy analysis of lysosomal / mitochondrial co-localisation. Results show that solar radiation exposure increases total lysosomal and mitochondrial mass and their colocalization, indicative of mitophagy. The molecular mechanisms of glutamine and melanin-induced mitophagy inhibition in skin cells exposed to solar radiation remain poorly understood. The regulators of mitochondrial turn-over upon exposure to solar radiation and their association with glutamine and melanin availability, as well as their signal transduction mechanisms remain to be fully elucidated and warrant further investigation.

4.5. Final Considerations

Mitochondria are highly dynamic organelles which convert one form of energy to another, providing the energy needed by specific cellular processes such as cell division, repair, and apoptosis. In the present study it was observed that mitochondria respond to low SSR in a cell type, time-lapse and dose-dependent manner.

Nevertheless, the description of changes in mitochondrial parameters in response to an important environmental stressor such as solar radiation can potentially identify intervention points for either increasing cellular defences against solar radiation or towards the expeditious removal of cells with potentially tumourigenic activity via apoptosis or autophagy (autophagic cell death).

Another important finding from the present study is that although mitochondrial mass changes are cell-density independent, parameters such as ROS production and MMP are sensitive to cell density-manipulations. The observation that some mitochondrial parameters such as MMP and deletion accumulation is presented in cycles of approximately 24 hours may be important in suggesting palliative interventions post solar radiation such as the use of antioxidants or antiinflammatory agents in order to reduce the solar radiation-associated damage to skin.

The induction of mtDNA lesions was found to be more pronounced with lower dose SSR and to peak 3 hours post irradiation. These findings further corroborate the hypothesis that mitochondria are more sensitive to solar radiation than nDNA. The highest incidence of mtDNA accumulation observed at 3 hours post irradiation may indicate that mtDNA repair mechanisms are initiated from several hours post solar radiation (between 3 and 24 hours).

Interestingly an association between increased mtDNA damage and reduction in mitochondrial mass may suggest either a reduction in mitochondrial biogenesis

rates or an increase in mitochondrial recycling. The reduction in mitochondrial mass in this case seemed to be associated with a mitochondrial response towards mtDNA repair.

Further observations from the present study include that low dose SSR increased lysosomal mass, possibly through lysosome biogenesis. Such organelles have important roles in protein and organelle recycling as well as reducing cellular energy expenditure through recycling of molecules such as amino acids and lipids.

The synchronised increase in mitochondrial and lysosomal mass post SSR is important given that the inefficient recycling of damaged mitochondria would have serious consequences for cellular and tissue homeostasis allowing the accumulation of potentially tumourigenic mutations in mtDNA. Further studies focusing on the interplay between mitochondrial and lysosomal adaptations in response to environmental stressors may significantly advance our understanding of skin carcinogenesis.

Importantly, the role of the cellular energetic source in the mitochondrial morphology adaptations and in the mitophagy induction is of great relevance given that it may indicate the energetic source of choice in responding to solar-induced damage to skin cells. For example, glutamine supplementation was observed to induce mitochondrial fusion, increased MMP and reduced mitophagy in A375 cells post SSR. The increase in energetic levels associated with glutamine supplementation is not as important as its possible effects in increasing

mitochondrial fusion, mtDNA repair and mitochondrial metabolism. Such conclusions were drawn from the fact that the reduced mitophagy levels, post SSR in glutamine supplemented tumour cells, were higher than that observed in the glucose supplemented cells.

These observations should not be extrapolated to human *in vivo* application nor be suggestive of a topical formulation supplemented with glutamine to confer mitochondrial fitness post solar radiation damage given that: (1) the glutamine effects were observed in tumour cell lines and its effects upon the human skin microenvironment containing both healthy and potentially tumourigenic cells are not known; (2) topical application or nutritional intake of antioxidants and supplements as solar-radiation protecting agents are controversial and subject to numerous confounding variables such as dosage, absorption rate, skin type, metabolic status and the subjects overall heath condition; (3) primary human skinderived cells such as keratinocytes, melanocytes and 3D skin models would be the next logical platform for follow-on investigations.

The present study clarified several mitochondrial responses to SSR in *in vitro* cell models of significant contribution to the field of mitochondrial biology and poses new questions related to mitochondrial adaptation to solar radiation.

Taking into consideration that mitochondria are possibly endo-symbiotic organelles and that they have developed highly organised responses to environmental stressors, particularly solar radiation, during the millions of years

in which the prokaryotic mitochondrial ancestor would have been a free living organism, it must be kept in mind that the symbiotic relationship between mitochondria and the eukaryotic nucleus as well as between mitochondria and other cellular organelles can themselves be potential targets of solar radiation effects observed in skin cells.

It is also possible that mechanisms presently considered reflect mitochondrial damage (reduction in MMP and mitochondrial mass, mtDNA deletions and point mutations) may yet be considered mitochondrial adaptations to their ever changing eukaryotic cell environment which, in its turn, is also constantly adapting to external stressors. A reduction in mitochondrial activity may be necessary to maintain the integrity of the eukaryotic cells through avoiding ROS damage. Similarly the presence of mtDNA deletions may reflect the plasticity of mtDNA and its adaptation to environmental stressors, given that cells can tolerate high levels of mtDNA before any loss in metabolic capability is sustained (mtDNA damage threshold).

It may also be possible that the presence of damaged mitochondrial genomes may have important roles in controlling mtDNA replication and translation. MtDNA damage could possibly serve as a 'cytoplasmic memory' to injury which in association to other cellular mechanisms such as telomere shortening would be important in controlling cellular life span.

Although highly speculative, disruption of the mitochondria-nucleus genetic intercommunication would potentially impact the ability of the cell to respond to environmental stressors. Once the symbiotic association between mitochondria and eukaryotic cells is successful to such an extent that the same is present in almost every eukaryotic living organisms it is reasonable to suspect that disruption of the normal communication between the symbionts would be a cause of molecular and cellular damage as well as of human diseases.

Human skin represents the first and the main barrier against solar radiation and the mitochondria of skin are the most exposed to solar radiation. Skin mitochondria may constitute the most important model of mitochondria and nucleus communication disruption elicited by a well studied environmental stressor. Detailed studies of gene expression control, cellular metabolism, damage repair as well as of mechanistic description of nuclear to mitochondria retrosignaling would not only constitute a new research field but also a potentially revolutionary one, in the sense that lessons learned from its mechanisms of disruption could reveal new and innovative ways of improving its communication, which in turn would be most beneficial to disciplines as diverse as skin cancer research to drug design and beyond.

Chapter 5: References

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Chapter 6 - Appendix

Awards received

Federation of the Societies of Biochemistry and Molecular Biology (FEBS) - Youth Travel Grant to attend the Mitochondrial Physiology Conference – The many functions of the organism in our cells, Obergurgl, Tyrol, Austria, 27th September – 01st October, 2010.

Conference Talks

Irish Radiation Research Society Scientific Meeting 2009 - 16-17 October - National University of Ireland, Galway - "Mitophagy in human skin cells post simulated solar radiation".

V Annual Meeting of the Irish Cytometry Society - 17-18 November 2009 - Science Gallery - Trinity College Dublin - "Nucleoids Remodeling and Impaired Mitochondrial Dynamics in Human Melanoma Cells in Response to UVR".

Scientists in Training Day – 08 February 2010 – Gray Institute for Radiation Oncology and Biology – University of Oxford – "Natural solar radiation damage to human skin mitochondria".

7th Mitochondrial Physiology Conference – FEBS Workshop: The many functions of the organism in our cells – 27. Sep – 1. Oct 2010– Obergurgl, Tyrol, Austria.-'Solar radiation damage to human skin mitocondria'.

Publications

- Zanchetta LM, Kirk, D Walsh J, Lyng FM, and Murphy JEJ, (2010) Cell density-dependent changes in mitochondrial membrane potential and ROS production in human skin cells post simulated solar radiation. Photodermatology, Photoimmunology and Photomedicine. (Published)
- Zanchetta LM, Walsh J, Lyng FM and Murphy JEJ (2011)- Mitophagy and altered mitochondrial dynamics in human melanoma-derived cells post exposure to simulated solar radiation. International Journal of Radiation Research (Published)
- Zanchetta L, Walsh JE, Lyng FL and Murphy JEJ (2011) Mitochondrial DNA Oxidative Damage and Repair dynamics post simulated solar radiation irradiation. (J. Invest. Dermatol.) (Submitted)
- 4. Zanchetta LM, Walsh J, Lyng FM and Murphy JEJ (2011)- Assessment of Simulated Solar radiation Irradiation effects on cellular and mitochondrial endpoints in spontaneously immortalized keratynocytes and human melanomaderived cell lines (In preparation)
- 5. Zanchetta LM, Walsh J, Lyng FM and Murphy JEJ (2011) Mitochondrial damage and DNA deletions in human keratinocytes and melanoma-derived cells induced by simulated solar radiation. (In preparation)
- Zanchetta LM, Walsh J, Lyng FM, and Murphy JEJ, (2011) Mitochondrial Nucleoid dynamics in human melanoma-derived cells post exposure to sublethal doses of simulated solar radiation. (In preparation)

Peer Reviewed Conference Abstracts

1) Zanchetta, LM; *et al.* (2009) - V Meeting of the Irish Cytometry Society, Dublin, Ireland. 'Nucleoid remodelling and impaired mitochondrial dynamics in human melanoma cells in response to UVR'.

2) **Zanchetta, LM**; et al. (2009) - 10th Annual Research Conference - The Research & Education Foundation at Sligo General Hospital, Sligo, Ireland. 'Mitophagy in human skin cells post simulated solar radiation irradiation'.

Zanchetta, LM; et al. (2009) - 4th All Ireland Cancer Conference, Dublin, Ireland.
 'Evaluation of mitochondrial membrane potential in response to solar radiation-induced ROS in melanoma skin cells'.

4) Zanchetta, LM; *et al.* (2009) – Mitochondrial Dynamics and Physiology. Whistler, British Columbia, Canada. 'Mitophagy and altered mitochondrial dynamics in human melanoma-derived cells post exposure to simulated solar radiation'.

Zanchetta, LM; et al. (2008) - 15th European Bioenergetics Conference (EBEC 2008). Trinity College Dublin, Ireland

6) Zanchetta, LM; et al. (2008) – European Meeting on Mitochondrial Pathology (EUROMIT 7), Stockholm, Sweden.

7) Zanchetta, LM; et al. (2007) – 13th International Congress of Radiation Research, San Francisco, USA.

8) Zanchetta, LM; et al. (2007) – Radiation Damage in Biomolecular Systems (RADAM07), Dublin, Ireland.

 Zanchetta, LM; et al. (2007) – Association of Radiation Research Annual Scientific Meeting, Belfast, Northern Ireland.

Other Conference Participations

1) 7th International Society for Stem Cell Research Annual Meeting. Centre Convencions Internacional. Barcelona. Spain. (2009)

IV Meeting of the Irish Cytometry Society. University College Dublin. Ireland.
 (2008)

3) 11th Institute of Molecular Medicine Meeting. Trinity Centre for Health Sciences. St.
 James Hospital. Dublin. Ireland. (2008)

 4) Oxidative DNA Damage – National Centre for Sensor Research – Dublin City University – Ireland. (2008)

5) I Nanotechnology Symposium: implications for human health, the environment and food safety. Dublin. Ireland. (2007)

6) 9th Institute of Molecular Medicine Meeting. Trinity Centre for Health Sciences. St. James Hospital. Dublin. Ireland. (2006)

7) I Latin American School of Human and Medical Genetics (RELAGH) – Porto Alegre. Brazil. (2006)

Undergraduate student co-supervision

1) Final year project. Project: Solar radiation damage to skin mitochondrial DNA and protective properties of melanin. Niamh M. Waters. BSc Biomedical Science. Department of Biological Science. Dublin Institute of Technology.

2) Ureka Program Student (2007). Project: Irish Solar radiation Damage of Human Skin Cell and Mitochondrial DNA. Piotr Chimielarz. Third year student of Biotechnology – Faculty of Biochemistry, Biophysics and Biotechnology – Jagiellonian University - Krakòw – Poland.

3) Ureka Program Student (2008a). Project: Mitochondrial DNA Damage and Repair assessment in melanoma cells post Simulated Solar Radiation. David Kirk. Final Year Student of Bachelor of Science (Chemistry and Biology) – National University of Ireland – Maynooth.

4) Ureka Program Student (2008b). Project: Mitochondrial DNA damage in melanoma and NMSC biopsies of tumoural lesions. Sophia Tappanelli. Second Year Student of Biological Sciences - Universita di Bologna. Italy.

5) Research Assistant (2009-2010) - Clodagh Kivlehan - 1st Class Honours in Biochemistry from NUI Galway. mtDNA studies in a cohort of Polymorphic Light eruption (PLE) patients.